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We regret that we could publish only two issues of the Annals of Biochemistry and Experimental Medicine during the year 1948 due to circumstances beyond our control. The Annals will appear regularly in the future.

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SOME OBSERVATIONS ON THE EFFECT OF SULPHANILAMIDE DERIVATIVES ON THE DEHYDROGENASE SYSTEM OF RESTING *E. COLI*

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(Received for publication, September 26, 1946)

That sulphanilamide derivatives act by competing with metabolites (1, 2) or interfering with certain bacterial enzyme systems, particularly the respiratory enzymes (3), is now generally accepted. It is also observed during chemical reactions that one of the hydrogen atoms of the—SO_2 NH_2 group in sulphanilamide is very reactive. As a matter of fact, various compounds have been obtained by replacing this hydrogen by suitable groups. This change has not only enhanced the bacteriostatic activity of the compounds concerned but has also widened the range of activity against various organisms. Indeed, it seems that this particular hydrogen atom may be the factor in determining the activity of the compounds.

Quastel showed that bacteria in their resting phases, do not decolourise methylene blue, but in the presence of succinic acid and other substances (which by themselves have no such action) they are powerful decolourisers. Obviously the resting bacteria cause certain changes to occur in the substances so that the latter donate hydrogen, which, in turn, reduces methylene blue.

In order to study the reactivity of the mobile hydrogen atom of the—SO_2 NH_2 group, a number of experiments has been made with sulphanilamide and some of its derivatives on the dehydrogenase activity of *Escherichia coli,* using the organism in the resting phase.

Experimental

The technique employed in determining the reduction of methylene blue by test substances was essentially that of Quastel (*vide supra*) with slight modifications. In preliminary experiments, it was found that young cultures did not reduce methylene blue even in the presence of succinic acid. On the other hand, cultures of more than 7 days' old were powerful hydrogen donators even without succinate (*vide* Table I). Ultimately, emulsions were prepared from 48 hours' old culture of *B. coli* in tryptic digest broth, after centrifuging and repeatedly washing the culture with normal saline. Firstly air and then nitrogen was passed through the final emulsion just according to Quastel. Opacity of the emulsion was determined by means of Brown's opacity tubes and the volume was so adjusted with normal saline as to compare with an opacity of 8. The emulsion was then preserved in the refrigerator. One per cent. solutions of sulphanilamide, sulphacetamide and sulphanilyl-benzamide in water were prepared. Sulphapyridine was made up to a strength of 45 mg. per cent. Mixtures for assay were made in Thunberg's tubes, with Keilin's modification. Vacuum was created by connecting the tubes with a "cenco" pump for 5 minutes. Methylene blue was placed in the side tube and added only when proper vacuum had been attained. The tubes were incubated in a thermostat at 45°. A control experiment without the addition of the drugs was done with each set of experiments. The results of the experiments are recorded in Tables I and II. The figures are the averages of at least three experiments.

TABLE I

Dehydrogenase activity of cultures at different periods of time.

Emulsion from tryptic broth culture of *E. coli* after	Constituents of mixture in Thunberg's tubes in cc.						Time of decolourisation in minutes
	Opacity	Emulsion.	Phosphate Buffer (pH. 7.2)	Methylene blue (1 : 5000)	Na-succinate M/100	Saline	
24 hrs.	8	1.0	2.0	0.5	1.0	2.0	No change
		1.0	2.0	0.5	Nil	3.0	,,
48 hrs. (1)	8	1.0	2.0	0.5	1.0	2.0	13.1
		1.0	2.0	0.5	Nil	3.0	No change
(2)	9	1.0	2.0	0.5	1.0	2.0	10.1
		1.0	2.0	0.5	Nil	3.0	No change
(3)	8	1.0	2.0	0.5	0.5	2.5	15
		1.0	2.0	0.5	Nil	3.0	No change
144 hrs. (1)	8	1.0	2.0	0.5	Nil	3.0	42
	8	1.0	2.0	0.5	Nil	3.0	25
288 hrs. (1)	32	1.0	2.0	0.5	Nil	3.0	13
		0.5	2.0	0.5	Nil	3.5	23
(2)	8	1.0	2.0	0.5	Nil	3.0	45
		1.0	2.0	0.5	1.0	2.0	10

TABLE II

Dehydrogenase activity of resting E. coli in the presence of sulphonamide derivatives.

Concentration of sulphanilamide, sulphacetamide and sulphanilyl-benzamide 1 per cent. Sulphapyridine-45 mg. per cent. Bacterial emulsion from 48 hrs.' culture. Opacity 8.

Methylene blue put in the side limb of the Thunberg's tube. Each determination of the time of decolorisation repeated at least 3 times or more.

Drug tested.	Constituents in Thunberg's tube in cc.						Time of decolourisation in minutes.
	Drug solution.	Emulsion.	Buffer ($pH=7.2$)	Methylene blue 1:5000.	Na-Succinate M/100.	Saline.	
Sulphanilamide 1%	1.0	1.0	2.0	0.5	...	2.0	15-23
	2.0	1.0	2.0	0.5	...	1.0	10-11
Sulphacetamide (sodium salt) 1%	1.0	1.0	2.0	0.5	...	2.0	13-17
Sulphanilyl benzamide 1%	1.0	1.0	2.0	0.5	...	2.0	12-15
Sulphapyridine 45 mg.%	1.0	1.0	2.0	0.5	...	2.0	No reduction
	2.0	1.0	2.0	0.5	...	1.0	,,
	3.0	1.0	2.0	0.5	...	...	,,
Positive control with Na-succinate	...	1.0	2.0	0.5	1.0	2.0	9-14
	...	1.0	2.0	0.5	...	3.0	No reduction
Negative control	...	2.0	2.0	0.5	...	2.0	,,

From Table II, it was evident that sulphapyridine did not cause any decolourisation of methylene blue. Rather, it prevented the slight lowering of the intensity of colour which usually occurred with all negative controls. It was, therefore, considered worthwhile to study its effect on a bacterial emulsion of high dehydrogenase activity. An emulsion from 6 days' old culture of *E. coli* was prepared, which decolourised methylene blue in 25-30 minutes. The requisite quantity of sulphapyridine solution instead of methylene blue was now placed on the side limb of the Thunberg's tube. Mixture of emulsion, buffer, saline and methylene blue was prepared in the tube and the latter put in the bath at 45°, after creating proper vacuum. When the colour of methylene blue, was discharged, the solution of sulphapyridine, kept in the side tube was poured in, and the time noted for bringing back the colour to its original intensity. Similar experiments with sulphanilamide were also conducted. The results are summarised in Table III.

TABLE III

Effect of sulphapyridine and sulphanilamide on the dehydrogenase activity of E. coli.

Mixture of ingredients in Thunberg's tubes in same proportions as in Table II. Drugs put in side limb, and poured after decolourisation.

Drug and strength.	Amount mixed in cc.	Time of decolourisation by bacterial emulsion.	Time of recolourisation of reduced methylene blue by drug.
Sulphapyridine 45 mg.%	0.5	27 min.	42 min.
	1.0	28 ,,	27 ,,
	2.0	27 ,,	18 ,,
Sulphanilamide 1%	1.0	26 ,,	No change
	2.0	27 ,,	No change

Discussion and Conclusion

From the results of the observation carried so far, it is apparent that sulphanilamide, sulphacetamide and sulphanilyl-benzamide serve as hydrogen donators in the presence of resting *E. coli*. Though not widely so, sulphanilyl-benzamide appears to be stronger than sulphanilamide in this respect, and sulphacetamide in between the two (Table II). But the action of sulphapyridine is found to be just the opposite. Here, the drug not only fails to reduce the methylene blue in the system, but on the contrary, oxidises it from a reduced solution (Table III). Such a phenomenon is however not observed with sulphanilamide. Obviously reduction of sulphapyridine in one case and oxidation of sulphanilamide (and its acyl derivatives) in the other is involved. This phenomenon may be interesting in explaining the mode of action of sulpha drugs. Oxidation of sulphonamides in the system prior to its exertion of bacteriostatic action was accepted at one time (4). This theory is, however, not accepted at present (5). Whatever may be the mode of action of these drugs, it seems certain that, in an anaerobic system, sulphanilamide and its acyl derivatives act as hydrogen donators, while sulphapyridine serves as an acceptor of hydrogen. This suggests that the probable mode of action of sulphapyridine may be different from that of sulphanilamide and its acyl derivatives (6, 7).

Further work will be taken up shortly to study the effect of other substituted derivatives of sulphanilamide on the dehydrogenase activity of *E. coli* and various other pathogenic organisms.

Summary

1. The effect of sulphanilamide, sulphacetamide, sulphanilylbenzamide and sulphapyridine on the dehydrogenase activity of resting *E. coli* has been studied.

2. While the compounds sulphanilamide, sulphacetamide and sulphanilylbenzamide are being found to reduce methylene blue in the system, sulphapyridine is observed to produce a contrary effect.

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BACTERICIDAL ACTION OF METALLIC COPPER ON *VIBRIO CHOLERAE.*

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(Received for publication, December 12, 1946)

Copper has long been known to have a lethal action on some of the minute organisms. Locke (1) in his experiments with distilled water from different sources showed that the water distilled in copper vessels had marked deleterious effect on tadpoles and tubifex and this he ascribed to the action of minute traces of copper present in that water. The bactericidal action of copper on *B. coli* and *B. typhosum* has also been studied by different workers. Kraemer (2) observed that *B. coli* and *B. typhosum* are completely destroyed by placing a clean copper foil in the water containing these micro-organisms, and advocated the use of copper foil as a domestic measure for ridding water of these pathogenic bacteria. Dougal (3), quoting Cushny, states that "certain organisms in water stored in a copper vessel are affected and killed by copper in solution so dilute that the metal is not detected chemically". But to other workers, the use of copper against *B. typhosum* and *B. coli* appeared unreliable.

A study as to how far this metal is really effective in water contaminated with *B. typhosum, B. dysentery* (Shiga), *B. coli* and particularly *V. choleræ* has been made.

EXPERIMENTS

Experiment 1.—This was conducted with distilled water *p*H 6·8 prepared in copper-free vessels. Eight flasks each containing 200 cc. of this water were

sterilised. Four pieces of thin copper foils ($\frac{3}{4}''$ square) were sterilised in hot-air steriliser. In this and in all subsequent experiments, the strips of copper were thoroughly cleansed and brightened with emery cloth before being sterilised. The sterile copper foils were aseptically transferred, one into each of the first four flasks and the remaining four flasks without copper were kept as control. One flask with copper and one without copper were respectively charged with 0·1 cc. of saline suspension of *V. cholerae, B. dysentery* (Shiga), *B. typhosum* and *B. coli,* all of which were recently isolated, smooth and virulent strains. The bacterial suspensions used in this and all subsequent experiments were freshly prepared from 18 hours' growth on Douglas agar slants, the bacterial contents being 5,000 millions per cc. of *V. cholerae* and 2,500 millions per cc. of the rest, so that the final bacterial contents in the flasks were 2·5 millions per cc. in case of *V. cholerae* and 1·25 millions per cc. in others. The flasks were kept at laboratory temperature and sterility tests were done from each flask (0·5 cc. inoculum separately in 30 cc. Douglas broth and in agar slant,—read after 48 hrs. incubation.) at half hour intervals for the first 8 hours and at gradually longer intervals during the later stage of the experiment. The results are given in Table I.

TABLE I

Substrate.	Micro-organisms tested.	Observation.
Distilled water *with* copper	*V. choleræ*	Dead after ½ hour.
Distilled water *without* copper	do.	Dead after 48 hours.
Distilled water *with* copper	*B. dysentery* (Shiga)	Dead after 3½ hours.
Distilled water *without* copper	do.	Dead after 5 days.
Distilled water *with* copper	*B. typhosum*	Dead after 20 hours.
Distilled water *without* copper	do.	Living after 15 days.
Distilled water *with* copper	*B. coli*	Dead after 20 hours.
Distilled water *without* copper	do.	Living after 15 days.

Experiment 2.—In this, instead of distilled water, ordinary tank water was taken. The water was collected from a local pond. For sterilisation, this was passed through Seitz-filter (E.K. 14) and aseptically transferred into sterile flasks in volumes of 200 cc. The *p*H of the water was 8·3 and the nitrogen content was found to be 0·4 mg. per 100 cc. (micro-kjeldahl). With this water the previous experiments were repeated. The results are given in Table II.

TABLE II

Substrate.	Micro-organism tested.	Observation.
Tank water *with* copper	*V. choleræ*	Dead after 1½ hours.
Tank water *without* copper	do.	Living after 15 days.
Tank water *with* copper	*B. dysentery* (Shiga)	Dead after 26 hours.
Tank water *without* copper	do.	Living after 15 days.
Tank water *with* copper	*B. typhosum*	Dead after 30 hours.
Tank water *without* copper	do.	Living after 15 days.
Tank water *with* copper	*B. coli*	Dead after 96 hours.
Tank water *without* copper	do.	Living after 15 days.

Experiment 3.—Sterile distilled water (*p*H 6·9) and two different samples of tank water No. 1 and No. 2, sterilised through Seitz filter were taken in three flasks. The *p*H. of both the samples of tank water was 8·3. The nitrogen content of sample of tank water No. 1 was 0·4 mg. per 100 cc. and of sample of tank water No. 2, 0·53 mg. per 100 cc. (microkjeldahl). Sterile copper strips ($\frac{3}{4}''$ square) were aseptically transferred, one into each of the three flasks. Three flasks of the corresponding samples of water without copper were kept as controls. 0·1 cc. of suspension of *V. cholerae* (5,000 millions per cc.) was added to water in each of these flasks. Sterility tests were done at 30 minute intervals. The results are given in Table III.

TABLE III

Water taken 200 cc. in each flask.

Substrate.	Micro-organism tested.	Observation.
Distilled water *with* copper	*V. choleræ*	Dead after ½ hour.
Distilled water *without* copper	do.	Dead after 48 hours.
Tank water No. 1 (total nitrogen 0.4 mg. per 100 cc.) with copper	do.	Dead after 1½ hours.
Tank water No. 1 *without* copper	do.	Living after 15 days.
Tank water No. 2 (total nitrogen 0.53 mg. per 100 cc.) *with* copper	do.	Dead after 3 hours.
Tank water No. 2 *without* copper	do.	Living after 15 days.

Experiment 4.—200 cc. of sterile tank water (*p*H 8·3; nitrogen—0·4 mg. per 100 cc.) were kept in contact with copper strips ($\frac{3}{4}''$ square) in each of three separate flasks. Copper strip was removed from one of the flasks after 2 hours and from another after 4 hours. The three flasks were then inoculated with 0·1 cc. of emulsion of *V. choleræ* (5,000 million per cc.). It was expected from the previous observations that water kept in contact with copper for several hours will have quick bactericidal action on *V. choleræ*. Accordingly, sterility tests were done at 5 minutes intervals. The results are given in Table IV.

TABLE IV

Substrate.	Micro-organism tested.	Observation.
Tank water from which copper strip was removed after 2 hours	*V. choleræ*	Dead after 30 min.
Tank water from which copper strip was removed after 4 hours	do.	Dead after 15 min.
Tank water from which copper strip was not removed	do.	Dead after 10 min.

Experiment 5.—With the same copper surface and increasing volumes of distilled water, the time for lethal action on *V. choleræ* was expected to be prolonged. Accordingly 50, 100 and 200 cc. volumes of sterile distilled water were taken in three different flasks. One sterile strip of copper ($\frac{1}{4}''$ square) was placed in each flask.

The flasks were charged with 0·025 cc., 0·05 cc. and 0·1 cc. of suspension of *V. choleræ.* (5,000 million per cc.) so as to make the final bacterial concentration equal in all the flasks. Sterility tests were done from each flask at half-hour intervals. The results are given in Table V.

TABLE V

Substrate.	Micro-organism tested.	Observation.
Distilled water 50 cc. *with* copper	*V. choleræ*	Dead after 3 hours.
Distilled water 100 cc. *with* copper	do.	Dead after 5 hours.
Distilled water 200 cc. *with* copper	do.	Dead after 6 hours.*
Distilled water 50 cc. *without* copper	do.	Living after 12 hours.
Distilled water 100 cc. *without* copper	do.	Living after 12 hours.
Distilled water 200 cc. *without* copper	do.	Living after 12 hours.

* With smaller strip of copper, longer time was required for complete lethal action (*cf.* Table I).

Experiment 6.—(a) Distilled water kept in contact with copper strip ($\frac{3}{4}''$ square per 200 cc. of water) for 48 hours was used in the preparation of peptone water (1 per cent.) tubes. These were inoculated with *V. choleræ, B. dysentery* (Shiga), *B. typhosum* and *B. coli* and were incubated at 37° for 24 hours.

(b) Douglas broth tubes in 20 cc. volumes with a copper strip ($\frac{3}{4}''$ square) in each tube were prepared. After 48 hours these were inoculated and incubated at 37° for 24 hours.

The results on culture and of subsequent viability are shown in Table VI.

TABLE VI

Substrate.	Micro-organisms tested.	Observation.
(*a*) 1% Peptone water (prepared in distilled water kept in contact with copper for 48 hours)	*V. choleræ*	Free growth, Living after 15 days.
	B. dysentery (Shiga)	do.
	B. typhosum	do.
	B. coli	do.
(*b*) Douglas broth with copper strip (used 48 hours after preparation)	*V. choleræ*	do.
	B. dysentery (Shiga)	do.
	B. typhosum	do.
	B. coli	do.

Experiment 7.—In this, in place of flasks with copper strips, thoroughly cleansed and brightened copper vessels were used. 200 cc. volumes of tank water passed through Seitz filter (E.K. 14) were taken in four such vessels. The *p*H of the water was 8·3 and nitrogen content was 0·46 mg. per 100 cc. The water in the vessels was charged separately with 0·1 cc. suspensions of *V. choleræ, B. dysentery* (Shiga), *B. typhosum* and *B. coli*, the final bacterial concentrations being the same as in the previous experiments. Sterility tests from each vessel were done at half-hour intervals. The results are given in Table VII.

TABLE VII

Substrate.	Micro-organisms tested.	Observation.
Tank water (200 cc.) in copper vessel	*V. choleræ*	Dead after $1\frac{1}{2}$ hours.
do.	*B. dysentery* (Shiga)	Dead after 3 hours.
do.	*B. typhosum*	Dead after 5 hours.
do.	*B. coli*	Dead after 5 hours.

Discussion

The experiments were repeated with different strains of the same micro-organisms and with different samples of distilled water and tank water. The *protocols* submitted are of typical experiments. The results clearly indicate that copper has got a definite bactericidal action on *V. choleræ, B. dysentery* (Shiga), *B. typhosum* and *B. coli* of which *V. choleræ* is one that is most readily affected. It was not possible to estimate the exact amount of copper that is just sufficient to kill these organisms. During these experiments, samples from both distilled water and tank water (200 cc.) kept in contact with copper ($\frac{3}{4}''$ square) for 24 to 48 hours have been frequently tested chemically for presence of copper, but the results have been uniformly negative. It has been observed that copper acts on these organisms more quickly in distilled water than in tank water containing organic nitrogenous matter (Tables I and II). With the increase of such substances in the water lethal action of copper on *V. choleræ* is delayed (Table III) ; and this action is found to be completely inhibited in peptone water and in Douglas broth, where the vibrios instead of being killed, survive and freely multiply (Table VI). This is possibly due to the fact that in such conditions, the concentration of copper can not reach its effective level because of its immediate combination with the organic constituents present in the substrate. With increase of volumes of water, either the copper surface or the time of contact had to be increased to have lethal effect on *V. choleræ* (cf. Tables, I, V and VII).

Lastly, in places where frequent epidemics of cholera occur and where the sources of drinking water are open tanks or wells exposed to the chance of contamination with *V. choleræ*, it is advisable to keep the water in clean copper vessels for several hours (4 to 6 hours) before use. Considering the possible bulk of contamination in usual natural water, this period of contact appears enough to kill any vibrio if present (Table VII). The advantage of such water over boiled water is that it is

resistant to any possible subsequent contamination in an infected household while in storage (Table IV). For prolonged storage water may be transferred to some other container after keeping in copper vessel for 4 to 6 hours. In absence of a suitable copper vessel, a few pieces of clean and brightened copper coins deposited in water in any container may serve the purpose.

SUMMARY

1. Metallic copper has got a marked bactericidal action on *V. choleræ*.

2. It also affects *B. dysentery* (Shiga), *B. typhosum* and *B. coli* but on these its action is not so quick as on *V. choleræ*.

3. During epidemics of cholera, in addition to inoculation of prophylactic cholera vaccine, use of clean copper vessels for storage of drinking water for a few hours is suggested.

We are thankful to Dr. T. N. Ghosh. D.Sc, Dr P. N. Basu. M.B. and Mr. S. N. Sen. M.Sc., for their kind suggestions and useful help in the work.

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THIAMIN CONTENT OF COMMON PUNJAB FOODSTUFFS IN THE RAW AND COOKED STATE

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(Received for publication, February 2, 1947)

In the course of our work on thiamin we had occasion to examine a large number of indigenous foodstuffs for their thiamin content, both in the raw state and after cooking according to methods used in Punjabi homes. These values are of interest not only to nutrition workers, from the point of view of nutrition and dietary studies, but are also of importance in illustrating the variations in vitamin content due to geographical and other environmental factors. At the same time the vitamin content of some of the foodstuffs listed have not so far been estimated in other laboratories in India.

The method of assay used was that described by Rattan and Ahmad (1) from this laboratory. The estimation of free and combined thiamin was made according to the technique described by Ahmad, Mehra, and Bharihoke (2). In Table I the thiamin content of the various foodstuffs both in the raw and the cooked state are shown. The cooked state implies the method of cooking commonly used in Punjabi homes. An outline of these methods for the more important foodstuffs are described below:

Wheat Chapatis.—Atta (93-95% extraction flour) is kneaded with the hands into a thick dough with a small amount of water in a flat metallic or earthenware vessel. The dough is allowed to stand for 1-2 hours. Small amounts of the dough are then beaten between the palms of two hands into thin round discs of 2-3 mm thickness and 10-20 cm diameter, using small amounts of dried atta on the palms to prevent the dough sticking to the hands. Chapatis in some homes are made by rolling the dough on a wooden plate with a small wooden roller instead of by hands. Hand-made chapatis are generally preferred. These are then baked on a hot iron plate, turning over once to bake both the sides equally. They are generally consumed while still hot.

Rice Pulao

Rice grains are washed in three changes of water to remove dust and extraneous matter, after which they are soaked in water for 1-2 hours. In a kettle some chipped onions are fried in fat until brown, when peas or any other vegetable is added followed by rice and finally a quantity of water which is generally twice the volume of rice to be cooked. The lid is then closed and the content allowed to

cook on a slow fire. Pulao is ready within 30-40 minutes and by this time the water added has all been evaporated or absorbed by the rice. No water is thrown away.

Pulse Curry

The pulse is first washed with three changes of water to remove all dust etc. and is then soaked in water for about half an hour. Water, about 4 times the amount of pulse taken is brought to boil in a kettle. To this are added curry powder and the pulse, and allowed to cook for about an hour. In a frying pan some chipped onions are fried in fat until brown and are added to the kettle on top of the curry. This may be brought to boil again if necessary.

Vegetable Curry

Some chipped onions are fried in fat until brown, when washed and chipped vegetables are added and the whole contents after the addition of some curry powder fried again for few minutes. Then water is added and allowed to cook for 30-40 minutes on a slow fire until ready.

TABLE I.

Thiamin content of common Punjab Foodstuffs in the raw and cooked state.

Name of foodstuff.	Thiamin content in μg per g. Raw state Free	Raw state Total.	Cooked state Free	Cooked state Total.	Loss in cooking %
I. *Cereals.*					
Wheat, (*Triticum vulgare*)					
,, 9-D	2.00	2.60	1.54	2.06	20.8
,, C-217	2.25	2.95	1.72	2.30	22.1
,, Type-4	2.24	3.20	1 86	2.46	23.1
,, Type-8	2.38	3.36	1.85	2.50	23.8
,, C-228	2.72	3.84	1.98	2.78	27.6
,, C-518	2.84	3.95	2.00	2.77	30.0
,, Type-1	3.52	4.64	2.28	3.11	33.3
,, C-591	3.63	4.84	2.35	3.20	33.3
Rice, (*Oryza sativa*), raw, unmilled.					
,, Jhona-349	—	3.33	—	1.07	64.9
,, Mahler 346	—	3.00	—	1.05	65.1
,, Palwan-sufaid	—	2.67	—	1.07	60.1
,, Mushkan	—	3.67	—	1.17	68.7
,, Sathra	—	2.67	—	1.07	60.0
,, Basmati	—	4.30	—	1.07	62.8
Oats, (*Avena sativa*)					
,, Type-T	5.35	5.35	—	—	—
,, Alagarian	5.35	5.35	—	—	—
,, Brunkaslo	4.95	5.00	—	—	—
,, Type Fos 1/29	5.00	5.45	—	—	—
,, Type French	4.00	4.25	—	—	—
,, I. P. Hyd.-3	5.00	5.00	—	—	—
Maize, (*Zea mais*)	1.82	2.13	—	—	—
Barley, (*Hordeum vulgare*)	2.04	2.52	—	—	—

TABLE I.—(*Contd.*)

Name of foodstuff.	Thiamin content in μg per g.				Loss in cooking %
	Raw state		Cooked state		
	Free	Total.	Free	Total.	
II. *Pulses.*					
Moth, (*Phaseolus aconitifolius*)					
,, T-9	—	4.96	—	—	—
,, T-10	—	5.00	—	—	—
,, T-4	—	6.01	—	—	—
,, T-16	—	6.67	—	—	—
,, T-711	—	6.67	—	—	—
,, T-3	—	5.98	—	—	—
,, T-1	—	5.90	—	—	—
,, T-7	—	5.67	—	—	—
Green Mung, (*Phaseolus Mungo*)	2.70	2.80	1.53	1.53	45.33
Black-gram, (*Phaseolus radiatus*)	3.17	3.50	1.26	1.59	54.20
Arhar, (*Cajanus Indica*)	2.50	3.00	1.20	1.50	50.00
Lentil, (*Lens esculentum*)	2.50	2.85	1.20	1.50	47.40
Bengal gram, (*circer arietinum*)	4.20	4.20	1.90	1.97	53.30
Dry peas, (*Pisum sativum*)	4.13	4.97	—	—	—
III. *Vegetables.*					
Green peas, fresh, (*Pisum sativum*)	2.30	2.45	1.50	1.50	38.78
Ghiya, (*Cucurbita lagenaria*)	1.30	1.50	0.80	0.80	46.67
Kadu, (*Cucurbita pepo*)	1.15	1.17	—	—	—
Tinda, (*Citrullus fistulosum*)	2.50	2.50	1.00	1.00	60.00
Potato, (*Salanum tuberosum*)	1.50	1.70	1.05	1.05	38.21
Spinach, (*Spinacia oleracea*)	3.50	3.65	2.70	2.73	25.25
Beans, (*Phaseolus Sp.*)	3.10	3.10	—	—	—
Ladys Finger, (*Hibuscus esulentus*)	0.63	0.65	0.40	0.42	35.38
Brinjals, (*Solanum melongea*)	1.00	1.20	0.60	0.62	48.33
Bitter gourd, (*Momordica Charantia*)	1.20	1.35	0.80	0.82	39.26
Tomato, (*Lycopersicum esculentum*)	0.85	0.85	—	—	—
Cabbage, (*Brassica Oleracea Capitata*)	1.70	1.80	1.10	1.15	36.11
Arvi, (*Colocasia antiquorum*)	2.00	2.30	1.20	1.20	47.82
IV. *Fruits.*					
Leechi, (*Litchi chinensis*)	—	0.40	—	—	—
Peaches, (*Amydgalus Persea*)	—	0.90	—	—	—
Oranges, (*Citrus aurantium*)	—	1.00	—	—	—
Banana, (*Musa sapientum*)	—	1.50	—	—	—
Pears, (*Pyrus Communis*)	—	1.00	—	—	—
Falsa, (*Grewia Asiatica*)	—	0.56	—	—	—
Mango, (*Mangifera indica*)	—	0.60	—	—	—
Apple, (*Pyrus malus*)	—	1.20	—	—	—
Strawberry, (*Fragaria grandiflora*)	3.10	3.20	—	—	—
V. *Miscellaneous.*					
Yeast, (*Saccharomyces cerevisæ*)	—	35.90	—	—	—
Tea, (*Camellia sinensis*)	—	0.00	—	—	—
Coffee, (*Coffea Arabica*)	—	0.00	—	—	—
Almond, (*Prunus amygdlis*)	—	2.50	—	—	—
Cocoanut, (*Cocos noucifera*)	—	1.05	—	—	—
Wallnut, (*Juglans regia*)	—	4.50	—	—	—
Raisins, (*Ribes rubrum*)	—	0.80	—	—	—
Ground-nut, (*Aracuis hypogea*)	—	5.20	—	—	—

In a second experiment, the tryptic digestion was preceded by peptic digestion for three hours. It was found that though the digestibility of soya milk protein by pepsin was slightly higher than that of cow's milk protein (8.2% for soya milk and 7.6% for cow's milk) the succeding tryptic digestion was much smaller for soya milk than for Cow's milk. Even after hydrolysis for 24 hours, soya milk was only about half as much digested as cow's milk. Extension of the hydrolysis with the ereptic enzymes of the small intestines did not increase the digestibility of the soya milk proteins to a level comparable with that of cow's milk proteins. Under these conditions while cow's milk protein was digested to the extent of about 90%, soya milk was only digested to the extent of 46%. The above experiments, therefore, showed that the poorer *in vitro* digestibility of soya milk is due to the very slow action of trypsin on the protein of the milk.

Recently, Bowmann (5), Kunitz (6) and Ham *et al.* (7) have reported the existence of a powerful tryptic inhibitor in raw soya beans. Based on these findings experiments were made by us to see whether the low digestibility of soya milk by trypsin is to be traced to the presence of the trypsin inhibitor in the milk.

Preliminary experiments were made to see whether soya milk contained this inhibitor. 10 cc. of soya milk were added to 90 cc. cow's milk and the rate of tryptic digestion of the mixture was compared with that of pure cow's milk. The rate of digestion is presented below.

TABLE II.

	2 hrs.	4 hrs.	8 hrs.	18 hrs.
Cow's milk	46.4	68.9	82.4	87.8
,, containing 10% soya milk	19.6	24.8	26.8	33.4

Addition of even 5% soya milk reduced the digestibility of cow's milk in 24 hours from 86% to about 56%. The same inhibiting effect could be produced by adding to cow's milk, soyabean milk whey prepared by adding minimum amount of acid to soya milk and filtering the precipitated coagulum of protein and fat. Similar observations were made by using a pure casein solution as substrate in stead of cow's milk.

The above results indicated that soya bean milk contained a measurable portion of the tryptic inhibitor present in the soya bean. Even if a large concentration of enzyme was added to soya milk, the effect of this inhibitor could not be counteracted. A typical result obtained in an experiment where the enzyme used in the case of soya milk was twice the amount used in the case of cow's milk is given below.

TABLE III.

	1 hr.	2 hrs.	4 hrs.	24 hrs.
Cow's milk	45.4	60.2	77.9	85.5
Soya milk	16.5	24.4	34.6	65.3

A further confirmation of the fact that the inhibitor was the cause for the poor *in vitro* digestion of soya milk was made by isolating the proteins from soya

and cow's milks and studying their relative digestibility by pepsin and trypsin. It was found that they were digestible to about the same extent as shown below:—

TABLE IV.

	Peptic	Tryptic		
	2 hrs.	2 hrs.	4 hrs.	24 hrs.
Cow's milk protein	9.2	39.9	43.6	49.5
Soya milk protein	9.1	38.1	42.4	47.4

The effect of autoclaving on the digestibility of soya bean milk was also studied. While making this study, simultaneous observations on the effect of autoclaving on the inhibitor present in the soya bean milk whey were also made. Soya milk and the whey prepared from it were autoclaved for 15 minutes at 10 lbs. pressure, and another batch at 20 lbs. pressure. The results are tabulated below.

TABLE V.

Effect of autoclaving on the digestibility of soya milk and the destruction of the inhibitor in soya milk whey.

	2 hrs.	4 hrs.	24 hrs.
Cow's milk	60.4	78.9	91.6
Soya milk autoclaved at 10 lbs.	27.2	36.9	54.3
Soya milk autoclaved at 20 lbs.	47.9	69.4	83.2
Cow's milk containing 10% whey autoclaved at 10 lbs.	29.6	39.4	59.7
Cow's milk containing 10% whey autoclaved at 20 lbs.	59.4	78.4	91.6

The above results showed that practically all the inhibitor was destroyed by autoclaving at 20 lbs. for half an hour. The inhibitor was quite active even after being autoclaved at 10 lbs. for 15 minutes. Corresponding with the destruction of the inhibitor, the digestibility of soya milk is also improved, but the above results show that the improvement in the digestibility is not as fast as the destruction of the inhibitor. The results are, nevertheless, fairly striking.

The available evidence would show that soyabean contains a very powerful tryptic inhibitor which resists the action of heat to a considerable extent. Further work was done to study the degree of destruction of the inhibitor present in soya-bean by various types of heat treatment.

Cold solvent extracted soyabean powder was extracted at *p*H 4 and the clear extract obtained after centrifuging was used as a source of the inhibitor. Since the activity of the inhibitor was very high, the extract had to be diluted very much (one g. of bean in 200 cc. of the extract) in order to study difference in the degree of destruction after heat treatment. This diluted extract was adjusted to *p*H 7 and autoclaved at 5, 10, 20 and 25 lbs. pressure respectively for half an hour. The activity after autoclaving was studied by adding a known amount of

the extract adjusted to *p*H 7.8 to cow's milk and studying the rate of digestion of the mixture with trypsin.

When 2 cc. of the inhibitor solution were added to 25 cc. substrate, the inhibiting effect could not be observed to a measurable extent in the case of the extracts autoclaved at 25 and 20 lbs. pressure. Slight inhibiting action could be observed in the case of extracts autoclaved at 10 and 5 lbs. pressure respectively. When 5 cc. of the extract was added the inhibiting effect was measurable. The results of the digestion of cow's milk after addition of the autoclaved extracts are tabulated below.

TABLE VI.

25 cc. milk at pH 7.8 + inhibitor solution at pH 7.8 + pepsin.

Heat treatment given to the inhibitor soln.	Vol. of inhibitor solution added.	Amount of milk protein hydrolysed in terms of N/100 NaOH.	Activity of inhibitor.	Percentage of original activity remaining after autoclaving.
	0	5.30	—	—
Autoclaved at 25 lbs.	5 cc.	5.25	0.05	0.13
,, 20 lbs.	,,	5.13	0.17	0.43
,, 10 lbs.	,,	4.87	0.43	1.08
,, 5 lbs.	,,	4.36	0.97	2.43
Merely boiled	,,	3.88	1.42	3.56
Raw extract not heated	0.25 cc.	3.32	1.98	100%

The above results suggest that practically all the inhibitor is destroyed on autoclaving at 25 or 20 lbs. for half an hour, while about 3.5% of the activity remains after boiling for half an hour. The activity of the inhibitor becomes progressively smaller as the pressure at which it is autoclaved increases.

Discussion

All the foregoing results clearly indicate that the poor *in vitro* digestion of soya milk is due to the presence of the tryptic inhibitor in it. Although, after boiling, the inhibitor retains only 3.5% of its original activity, this amount of the inhibitor passes on to the milk and this interferes with the normal tryptic digestion of the milk protein under *in vitro* conditions. Under *in vivo* conditions, however, the soya milk protein is nearly as digestible as cow's milk. It is not known whether this apparent discrepancy between the *in vitro* and *in vivo* digestibility can be explained by the fact that under *in vitro* conditions, the amount of enzyme added is strictly limited, while under the *in vivo* conditions, both the activity of the enzyme secreted as well as the quantity of the digestive secretions are determined by the nature of the food ingested and depend on the needs of the system during the period of digestion. Experiments are in progress to see whether the above can provide a satisfactory explanation.

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IN VITRO DIGESTIBILITY OF GROUNDNUT MILK

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In a previous communication (1), the method of preparation of milk from groundnut and the study of its nutritive value have been described. By experiments on animals, it was found that groundnut milk protein was digested to about the same extent as that of cow's milk. A study of the *in vitro* digestibility of groundnut milk compared to that of cow's milk has been presented in this note. The digestibility of the protein of the milk by pepsin and trypsin both separately and successively and that of the fat by lipase have been studied. Cow's milk and the milky emulsion prepared from the raw groundnut have been used for comparison with the groundnut milk prepared from the germinated seeds.

The method followed for studying the protein digestibility was to digest the milk samples with the enzymes under optimal conditions of *p*H and temperature, the digestion being followed by determining the amount of protein soluble in 7% trichloracetic acid. The digestibility is expressed as the percentage of milk protein rendered soluble in 7% trichloracetic acid by the action of the proteolytic enzymes. The percentage digestibility of the milk proteins is presented in the following table.

TABLE I.

Enzyme used	Time in hrs.	Raw groundnut emulsion not heated.	Groundnut milk prepared from germinated groundnut.	Cow's milk
Pepsin	2	11.9	10.2	7.6
	4	17.8	15.4	9.3
	18	27.4	25.2	21.1
Trypsin	2	27.6	43.2	48.0
	4	30.8	52.4	64.2
	18	45.9	71.8	87.6
Pepsin	3	12.5	10.7	7.8
Pepsin followed by trypsin	2	41.2	44.6	47.2
	4	52.9	55.7	59.6
	18	67.7	72.6	76.7

The digestibility of the groundnut milk was slightly improved by autoclaving the milk at 15 lbs. pressure for 15 minutes.

The fat in the milk was digested by pancreatic lipase prepared according to Willstætter and Waldschmidt-Leitz (2), the extent of hydrolysis being measured by titrating in alcoholic medium the fatty acids liberated according to the method of Willstætter, Waldschmidt-Leitz and Memmen (3). The milk samples were suitably digested so as to contain exactly 3% fat. The milks were all buffered at *p*H 8 for studying the enzyme action. The extent of enzyme digestion as measured by the volume in cc. of N/10 alkali equivalent to the fatty acids liberated is given in Table II.

TABLE II.

Milk used.	Time in hours. 1 hr.	8 hrs.	4 hrs.	2 hrs.
Cow's milk	0.46	0.88	1.22	1.54
Groundnut milk from *germinated groundnut*	0.22	0.43	0.51	0.97
Raw groundnut emulsion	0.19	0.40	0.49	0.86

It will be seen from Table I that the digestibility of the milk proteins by trypsin alone is highest in the case of cow's milk while it is minimum in the case of the raw groundnut emulsion. Peptic digestion is faster in the case of the groundnut milk than in the case of cow's milk. The preliminary peptic digestion promotes a successive tryptic digestion. This effect is highly pronounced in the case of the groundnut milk samples and very little in the case of cow's milk. A similar observation was made by Adolph and Wang (4) while studying the *in vitro* digestion of soya milk. They found that soya milk protein is better digested by pepsin than cow's milk protein, while the reverse in the case with the tryptic digestion. They also reported that denaturation of the protein by heat favours tryptic digestion while retarding peptic hydrolysis. Exactly similar observations have been made in the case of groundnut milk.

The slightly higher peptic digestion in the case of the groundnut milk samples is perhaps to be explained by the fact that after acidification to *p*H 2 and addition of pepsin, cow's milk gets curdled into lumps while there is no curdling in the case of groundnut milk. The latter, being in the form of an emulsion, is liable to be acted upon faster by pepsin. As to whether the lower tryptic digestion in the case of groundnut milk is to be traced to the possible existence of a tryptic inhibitor similar to that found in soyabean as investigated by Ham (5), Kunitz (6) and Bowmann (7) is under investigation.

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STUDIES ON VITAMIN C IN SOYABEAN AND SOYAMILK

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Although a good deal of knowledge has now been gathered regarding some aspects of the nutritive value of soyabean and soyamilk (Desikachar *et al,* 1), little is yet known about the status of vitamin C in bean and the milk prepared therefrom, and about various factors governing it. Particularly no information exists about this aspect with regard to the comparative effect of different conditions on soyamilk as against cows' milk. The problem is of much interest when it is remembered that cows milk as such is a poor source of vitamin C from the view point of meeting the daily requirements of infants subsisting on it alone, and even that amount is not available often on processing due to the destruction caused by different agencies.

With a view therefore to elicit detailed information on this subject from various aspects present investigations were undertaken.

Experimental

Soyabeans from local market, both black as well as white varieties as also those imported from outside (Bengal) were employed in the studies. Germination of the beans was done, after initial overnight soaking in three times the volume of water, in a shallow enamelled tray between two layers of thick cloth which were kept wet by occasional sprinkling of water. The soyamilk was prepared from germinated soyabean as described earlier (2). Glass-distilled water was used for preparation of the soyamilk in order to eliminate additional complication due to copper catalysis. The effect of added copper on vitamin C in soyamilk is determined separately, as also the effect of using tapwater for milk preparation.

Estimations of vitamin C were made by the method of Harris and Oliver (3)—in case of milk with suitable alterations—with the addition where necessary of formaldehyde condensation procedure according to Mapson (4) for differentiating non-vitamin C reductants. As may be noted from Table II the percentage of these was quite considerable, especially in the earlier stages of germination. Their amount did not increase appreciably as the germination proceeded. However the reducing value of the raw soyabean seems to be entirely due to them and as such there is possibility of wrong inference when modification of estimation procedure is not resorted to. It was further seen that the non-vitamin C reductants belonged mostly to the group of sulphydral compounds which rapidly condensed with formaldehyde at *p*H 0·6. In the present investigations the modified procedure therefore consisted of bringing the *p*H of the extract to 0·6 with H_2SO_4, addition of 2 per cent. HCHO and after 8 minutes titration against the indophenol dye. Correction was applied for the minute amount of ascorbic acid which also condensed during the period. The

dehydroascorbic acid estimations were done as usual, by passing H_2S for about 20 minutes through the extract (serum in case of soyamilk) and removing it the following day by a current of nitrogen under suction.

Results

In Table I below are presented priliminary observations regarding the germination of soyabeans and the quality of soyamilk prepared therefrom after. different stages of germination.

Quality was based upon the observations pertaining to the taste, flavour and smell specially the absence of bitterness and the raw vegetable flavour. Keeping quality was judged as the time the milk could stand storage in refrigerator without undergoing deterioration and coagulation.

TABLE I

Germination of soyabeans and quality of soyamilk prepared therefrom.

Period of germination in hours after initial overnight soaking (18 hours).	Length of sprouts in cm.	Fresh weight of 100 seedlings in g.	Dry weight of 100 seedlings in g.	Quality of the soyamilk.	Keeping quality in days.
24	1.3	30.2	14.11	Good	4-5
48	2.6	32.6	15.0	Good	4
72	3.5	36.5	15.9	Fair	2-3
96	6.7	40.3	18.4	Bad	1
120	8.9.	42.8	19.9	,,	1
144	11.0	45.0	19.7	...	...

The factors responsible for affecting the keeping quality are probably the development of acidity and lipolytic enzymes consequent to extended germination. A milk of good quality and as may be seen from later tables of practically optimum vitamin C content is given by about 48-72 hours' germination. It also keeps well in a refrigerator for at least 3 days.

TABLE II

Presence and extent of non-vitamin C artifacts in germinated soyabeans and soyamilk.

Period of germination in hours.	Vitamin C as estimated by direct titration mg./100 g.	After formaldehyde condensation mg./100 g.	Amount of non-vitamin C artifacts estimated as vitamin C mg./100 g.	% of artifacts to the vitamin C values.
0 (dry seeds)	2.7	0.0	2.7	100
0 (soaked 18 hrs.)	5.9	4.1	1.8	33
24	11.5	9.5	2.0	17.4
48	15.2	13.0	2.2	14.5
72	16.6	14.8	1.8	10.8
Soyamilk prepared from 48 hours germinated beans. Values—mg. per lit.	21.6	20.5	1.1	5.1

Values expressed as total vitamin C, *i.e.*, reduced + dehydroascorbic acid.

The results of systematic study of the production of vitamin C—reduced, dehydro—and total ascorbic acid in germinating soyabeans and the values for soyamilk prepared therefrom at various stages of germination over a period of 6 days, are presented in Table III. It may be observed that the vitamin C value reaches more or less an optimum level at the end of 72 hours' germination period after which there is only a slight increase in the value. This is also reflected in the vitamin C values of the Soyamilk prepared from beans after sucessive germination periods. Beans germinated for 72 hours appear to be best from the viewpoint of a fair quality milk with practically maximum vitamin C value. However for a better quality those germinated for 48 hours would be more satisfactory. An interesting point of note is the comparative high percentage of vitamin C existing as the dehydroascorbic acid during the early stages of germination. In later germination stages this appears to be mostly converted to the reduced form. These observations are in keeping with the earlier findings with germinating green gram (Rangnekar, unpublished) and point to the possibility of dehydroascorbic acid functioning as the first precursor of vitamin C during its biosynthesis.

An appreciable part of the soyamilk is also in form of dehydroascorbic acid which is biologically active. However the order does not correspond with the figures of the germinated beans. It is quite probable that a large fraction of the dehydroascorbic acid existing in the germinated beans is degraded during the processing operation for milk preparation into further biologically inactive forms like diketogulonic acid and only a part remains as the active dehydroascorbic acid. A slight fraction of vitamin may also undergo oxidation and consequent destruction during the processing stages. However after initial trials, the conditions for the preparation of the soyamilk were so adjusted as to involve a minimum loss of vitamin C originally present. A detailed study of some of the factors effecting this is presented later on.

TABLE III

Production of reduced, dehydro- and total ascorbic acid in germinating soyabeans and the vitamin C values of the resulting soyamilk.

Stage of germination.	No. of hrs. of germn. after initial soaking.	"Soyabeans" Values expressed as mg./100 gm. Reduced.	Dehydro-.	Total.	Total vit. C expressed on dry wt. basis.	"Soyamilk" Values expressed as mg. per litre. Reduced	Dehydro.	Total
0	0*	2.2	2.3	4.5	8.9	4.8	1.6	6.4
I	24	5.2	4.1	9.3	19.9	11.2	4.5	15.7
II	48	9.4	4.2	13.6	29.4	17.6	3.2	20.8
III	72	11.6	3.6	15.2	34.8	21.1	1.5	22.6
IV	96	14.3	1.1	15.4	33.7	20.5	1.6	22.1
V	120	15.0	1.8	16.8	36.1	...	...	...
VI	144	14.2	1.5	15.7	35.9	...	...	...

* 18 hours' soaking.

Next the influence of varietial differences on the vitamin C production in the germinating soyabean and the values for soyamilk was investigated, so as to hit upon the variety most satisfactory from the point of maximum vitamin C content of the prepared milk. Results given in table IV show that the white variety as compared the black one yeilded much higher results. The one from Bengal was specially best suited. An added advantage with the white variety of soyabean is that during the milk preparation complete dehusking prevent introduction of any dark coloration to the milk.

TABLE IV

Varietal differences and vitamin C production in germinating soyabeans and the milk prepared therefrom.

Variety.	Viability expressed as % germination.	Period of germination hours after initial soaking.	Length of sprouts (cm.)	Total vitamin C in 100 g. of fresh beans (mg.)	Total vitamin C of the soyamilk mg. per litre.
Black					
(local)	71	24	0.9	7.1	...
		48	1.8	11.2	17.4
		72	2.5	12.4	19.6
Black No. 2					
(from Bengal)	78	24	0.8	8.6	...
		48	1.9	12.7	18.9
		72	2.7	13.7	...
Black No. 3					
(from Bengal)	80	24	0.8	7.9	...
		48	2.0	10.5	17.8
		72	2.7	12.8	21.0
White					
(local)	98	24	1.3	9.3	15.7
		48	2.6	13.6	20.8
		72	3.5	15.2	22.6
(from Bengal)	99	24	1.2	9.1	...
		48	2.8	14.8	21.7
		72	3.6	17.4	23.4

During initial studies, the germinating soyabeans were observed to possess considerable ascorbic-oxidase activity. Since the release of this during the preparation stages of the soyamilk from the germinated beans would greatly affect the final vitamin C values of the milk, a detailed study of this aspect was deemed essential. In table V below are presented results regarding development of ascorbicase activity on extended germination of the soya bean. For the determination, 4 g. of the germinated bean were thoroughly ground in glass water, centrifuged and made to 20 cc. to 10 cc. of the centrifuged extract were added to 3 cc. sorensen's phosphate buffer *p*H 6·0 and 2 cc. of ascorbic acid soln. containing 2 mg. of the vitamin and the mixture was then kept at 25° C. At stated intervals of time 2 cc. of the solution were withdrawn 5 per cent. HPO_3 added to stop the reaction and then titrated against stadardized indophenol dye. The results are extressed as percentage original ascorbic acid destroyed. Correction was applied for the ascorbic acid present in the germinated bean.

TABLE V

Development of ascorbic Oxidase in germinating soyabean.

Period of germination in hours.	% Destruction of ascorbic acid in time (mins.) 10	20	40	60
0 (Soaked 18 hours)	0	0	0	0
24	13.3	30.6	41.2	64.6
48	31.5	52.5	64.8	100.0
72	29.2	47.4	67.5	88.3
96	26.3	47.6	59.8	77.9

It may be noted that the activity reaches maximum at about 48 hours germination period and then falls down. As against this it may be recalled that Vitamin C production reaches near maximum value on 72 hours germination. Thus it would be apparent that ascorbic oxidase activity is an important factor in determining the vitamin C values of milk prepared from the beans germinated for 48 or 72 hours. During the soymilk preparation the time taken to reach the stage of boiling the mass—i.e., when most of the enzymic activity would be destroyed—is easily anywhere between 10-20 mins and as obvious from the above table, in case initial scalding is not restored to the loss due to enzymic oxidation may easily exceed 20 per cent. of the normal vitamin content. Preliminary scalding of the beans is therefore essential prior to the preparation of soyamilk. It also helps in removing the bitter principles and other factors which adversely affect the good flavour of soyamilk. However a proper adjustment of the conditions of scalding has to be brought about which would accomplish the complete inactivation of the ascorbicase while at the same time affecting to a minimum extent the vitamin C content of the beans and the milk prepared therefrom. Results of some experiments carried with this view are given in Table VI. The scalding of the germinated beans was done in an aluminum vessel heated and maintained at the requisite temperature. It also contained 0·04 per cent. sodium bicarbonate. Contrary to expectations there was not any significant extra loss of vitamin C in the bean on this account, as seen from a separate experiment (Table VII). The addition of 0·04 per cent. sodium bicarbonate helped in eliminating the bitter principles of the bean pericap and in improving the taste of the milk.

As may be seen from Table VII scalding at 75° for 10 mins is sufficient for bringing about almost complete inactivation of the ascorbic oxide while least affecting the vitamin C value of the milk. Higher temperatures inactivate the enzyme in shorter time but the deleterious effect on the vitamin C in the milk is considerable.

TABLE VI

Influence of hotwater scalding on ascorbic oxidase destruction and final vitamin C values of soymilk.

Activity expressed as % added vitamin C destroyed in 15 minutes.

Conditions of Scalding Temperature	Time in mins.	Ascorbic-Oxidase activity 48 hours. germinated beans.	72 hours. germinated beans.	Vitamin C values of the soyamilk. mg. per litre (from 72 hrs. germn. beans).
	5	8.4	10.7	16.8
75°	10	2.3	1.1	22.6
	15	nil	nil	19.4
	5	3.1	2.9	20.0
85°	10	nil	0.8	18.9
	15	nil	nil	16.6

TABLE VII

Effect of 0·04 per cent. sodium bicarbonate in the scalding water on the vitamin C values of germinated soyabeans and soyamilk.

Conditions.	Vitamin C content of soyamilk mg./litre.	Vitamin C content of germinated beans mg./100 g.
Scalding (10 mins. at 75°) in water alone	15.3	22.2
,, plus 0.04% sodi bicarbonate	15.1	21.9

Conclusions

A systematic study of the production of vitamin C in soyabean on extended germination, of its utilisation towards the preparation of soyamilk of high vitamin C content as compared to cows' milk and the relationship of other factors governing these two aspects has revealed that 72 hrs. germination of the beans (preferably the white variety) yields a fair quality milk containing nearly optimum amount of vitamin C—22·6 mg. per litre—which is appreciably higher than the average value given by cows' milk, provided an initial scalding is carried out at 75°C for 10 minutes to destroy the oxidase. It is also noted that the beans contain non-vitamin C reductions—mostly of sulphydral nature—percentage of which is considerable in the initial stages of germination. The existence of a larger proportion of vitamin C in the germinating beans during the early stages as dehydroascorbic acid and their conversion to reduced ascorbic acid in later ones is again observed in the present investigations.

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STABILITY OF VITAMIN C IN SOYAMILK AS COMPARED TO COWS MILK

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The high susceptibility of the vitamin C in cows milk to destruction from different agencies has been known since a long time. Particularly known has been that catalized due to Cu either present naturally or acquired later on from holding vessels—specially during pasteurization—and the resulting oxidised flavour (Sharp, 1, Hand *et al*, 2) found to be ineffective in the absence of oxygen—by deaeriation of the milk—the catalysis due to Cu was observed to be checked by heating at temperatures much higher than pasteurization ones, presumably through the inactivation of vitamin C-oxidising enzyme present in cows milk (2). Kon and Watson (3) first noted the deleterious effect of sunlight exposure on vitamin C in cows milk and later Hopkins (4) showed it as that caused by the photochemical sensitization due to lactoflavin present in milk which in visible light is itself decomposed into lumichromes capable of catalyzing the oxidation of ascorbic acid. It was thought that a detailed study of the stability of vitamin C in soyamilk as compared to cowsmilk under different conditions would be of great importance. Results of investigations are reported below.

In table I are presented the comparative stabilities of vitamin C in soyamilk and cowsmilk held at 5° C. It would at once be seen that the retention is much higher in the former as against the latter. Effect of added thiourea 0·01 per cent. which is known to be an excellent Cu—inhibitor and at the same time non-toxic, was also investigated. Its beneficial effect in lessening the vitamin C losses on storage, markedly in case of cowsmilk, is apparent. That preparation of soyamilk either in glass-distilled water (completely free from contaminated Cu) or in tapwater (contaminated with appreciable amount of Cu) does not make a very much difference in the Vitamin C losses may also be noticed, although however it is advisable to use glass-distilled water for preparation of soyamilk for experimental purposes. This indicates that the vitamin C in soyamilk is protected against catalysis due to small amounts of Cu introduced during the processing stages. The detailed studies on the effect of added high amount of Cu—to the extent of 0·15 mg. per litre (an amount which is easily introduced from holding methods of pastuerisation) on the vitamin C

stability in soyamilk and cowsmilk, are given in Table I. The very serious loss of original vitamin C in cowsmilk, to the extent of 94 per cent. in 3 days at 5° is apparent. On the other hand the corresponding loss in soyamilk is only about 53 per cent.—although much more than soyamilk alone. The marked protection offered by thiourea against Cu catalyzed destruction of vitamin C in both cowsmilk and soyamilk—although far more apparent in case of former (there is only about 57 per cent destruction in case of thiourea—stabilized cowsmilk as against 94 per cent. in case of the one without it) may also be observed.

The results for soyamilk clearly indicate that the vitamin C therein as against that in cowsmilk is effectively stabilized against destruction on storage specially due to Cu catalysis. The protection offered is however not optimum as could be seen from the further beneficial effect of added thiourea.

As may also be seen from Table II no support could be obtained for the observation of Sharp (5) that heating at high temperatures (77°) inactivates the capability of added Cu to catalyze the destruction of vitamin C in cowsmilk—presumably due to the inactivation a Cu—enzyme. Actually there was slightly more loss.

TABLE I

Vitamin C in soyamilk and cowsmilk stored at coldroom temperature (5°) and the stabilizing effect of added thiourea.

Description.	Original vitamin C content in mg. per litre.	Storage in days (% loss in Vitamin C) 1	2	3
Soyamilk alone (prepared in glass—distilled water)	22.4	8.6	17.5	22.5
,, + thiourea 0.01%	22.4	6.9	16.4	20.2
Soyamilk prepared in tapwater	21.3	10.1	17.4	24.5
Cowsmilk	17.3	15.4	22.4	36.8
,, +m thiourea	17.3	10.2	19.1	30.2

TABLE II

Destruction of vitamin C in soyamilk and cowsmilk due to Cu catalysis and its prevention by thiourea.

Sl. No.	Description.	Original vitamin C mg./litre.	% Vitamin C destruction in days (stored at 5°)		
(i)	Soyamilk alone	22.4	8.6	17.5	22.5
(ii)	,, +Cu 0.15 mg./lit.	22.4	17.3	29.7	52.6
(iii)	,, + ,, + thiourea 0.01%	22.4	13.5	21.8	30.0
(iv)	Cowsmilk alone	17.3	15.4	22.4	36.8
(v)	,, + cu 0.15 mg./lit.	17.3	38.6	77.1	93.5
(vi)	,, + ,, + thiourea 0.01%	17.3	20.6	34.7	56.5
(vii)	,, + ,, heated at 77° for 15 minutes before storage.	15.6	40.1	78.4	97.6

The results of investigations on the influence of sunlight exposure on the vitamin C destruction in soyamilk as compared to cowsmilk due to the photochemical sensitization caused by existing riboflavin are presented in Table III. It would at once be seen that whereas the losses in vitamin C in cowsmilk on exposure to direct sunlight for two hours was of the order of 90 per cent. it was extremely small only about 2-3 per cent.—in case of soyamilk exposed to direct sunlight for same period. That bringing up the level of riboflavin to that of cowsmilk—assuming that to be a prime factor in the photochemical sensitization—does not in any marked way induce additional vitamin C losses in soyamilk may also be seen from Table III.

TABLE III

Influence of sunlight exposure on vitamin C in soyamilk and cowsmilk and the destruction due to riboflavin—caused photochemical sensitization.

Description	Original Vitamin C content mg. per litre	% retention of the original ascorbic acid in time (minutes) 15	30	60	120
Cowsmilk	17.8	77.8	65.9	42.6	11.1
Control (kept in dark)	17.8	100.0	100.0	—	92.1
Soyamilk	21.9	100.0	96.4	97.3	97.3
,, Riboflavin content brought to the level of cowsmilk (addition of 25 μg. per 100 cc.)	21.9	100.0	95.8	98.0	96.5
Control (kept in dark)	21.9	100.0	100.0	—	100.0

The results clearly point out that the vitamin C in soyamilk is not only stabilized against long storage and Cu catalized destruction but also against that due to sunlight through sensitization by riboflavin. Final confirmation of this fact and the fact that the protecting factor or factors are absent in cowsmilk was sought from the observation that vitamin C in cowsmilk mixes with equal volume of soyamilk was more stable than cowsmilk alone under the above conditions.

TABLE IV

Stabilization of vitamin C in cowsmilk mixed with Soyamilk (1:1).

Description.	Vitamin C Original mg. per litre.	% destruction of original vit. C in time			
			1 day	2 days	3 days
"Cu Catalyzed (0.15 mg/l)"—					
Cowsmilk alone	18.1	—	35.9	80.1	95.2
Cowsmilk + soyamilk (1:1)	20.1	—	24.3	59.6	75.6
		15 mins.	30 mins.	60 mins.	120 mins.
"Induced on sunlight explosure"—					
Cowsmilk alone	18.1	20.9	37.1	60.2	91.2
Cowsmilk + soyamilk (1:1)	20.1	9.4	16.2	33.4	50.7

CONCLUSIONS

It has been known since a longtime that Cu and more specially sunlight is instrumental in causing a severe destruction of vitamin C in cowsmilk. The prevention of former at least, was suggested (Hand *et al*, 2) by means of a deaeriation process and heating above pasteurization temperatures. The destruction due to second agency—caused by lactoflavin present in milk—may also be prevented to some extent by this means as the vitamin C oxidation in second case is not known to proceed in absence of molecular oxygen. The present investigations have shown the beneficial effect of added thiourea—a nontoxic Cu inhibitor—in stabilising vitamin C against the first type of destruction, both in cowsmilk and soyamilk, although very markedly in the former case. The high stability of vitamin C in soyamilk against both types of destructive agencies has pointed out the presence of vitamin C protecting factor or factors in soyamilk which are absent in cowsmilk. With the evidence at hand, it is difficult to postulate the nature of protecting system. Some plants have been known to contain protective mechanism for vitamin C against Cu—Oxidation (5, 6) have demonstrated the presence of an enzyme responsible for reduction of dehydroascorbic acid to ascorbic acid in broad beans. However the protective factors in soyamilk could not possibly be enzymic in nature as it would not survive the high heating in the processing stages of soyamilk. The fact that the protective mechanism is completely able to inhibit the sensitization due to riboflavin which is decomposed into lumichromes (or other products) which in turn bring about the vitamin C oxidation would at first suggest that either the protective factor combines with lumichromes (or other decomposition products), thereby eliminating them or that it is oxidised at a rate faster than vitamin C itself. The latter possibility, however, would appear less when it is considered that when all of the factor is oxidised addition of riboflavin would cause destruction of vitamin C. The effect of added riboflavin, however, does not indicate this.

Further work which would throw light on the possible nature of the vitamin C protecting system in soyabean and soyamilk is in hand.

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A NUTRITIONAL STUDY OF DIFFERENT POPULATION GROUPS OF NORTHERN INDIA WITH RESPECT TO THIAMIN

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In two earlier papers (Ahmad, Mehra, and Bharihoke 1946, i and ii) the results of a careful investigation of the thiamin content of different kinds of wheat, wheat products, and other foodstuffs, constituting the main items of diet and furnishing the greater portion of thiamin in the dietary of the people in Northern India have been reported. In both these studies the loss of thiamin in cooking and in the making of various food preparations from cereals and cereal products, according to the methods commonly used in Punjabi homes were also carefully investigated. On the basis of this knowledge, it was considered desirable to determine the thiamin intake of different population groups in this region. This was of particular interest because this region is regarded as one of the large thiamin consuming areas, while on the other hand certain mild conditions which respond to thiamin therapy are not uncommon.

In this study the thiamin intake of 31 human groups at different economic levels with certain differences in dietary habits and living in different rural and urban parts of Northern India, have been investigated. The study is based on 31 dietary surveys, one of which was undertaken by the authors in Lahore, and 30 others published surveys by the Department of Public Health, Punjab, and other nutrition workers in the region. In most dietary studies the estimates of vitamin intakes are generally made on raw food basis, calculating values from data reported in published food tables. Obviously this method has limitation. We have, in this investigation, taken into consideration the thiamin content of the cooked food as consumed by the people by taking into account the losses in the indigenous method of cooking. At the same time the values of thiamin used were those which referred to the local varieties of foodstuffs grown in the region. In Table I, are presented

the description of the various population groups and the data regarding their average thiamin intake.

TABLE I.

No.	Description of the population group.	Total thiamin intake on cooked food basis (μg)	Total calorie intake.	Thiamin intake per calorie. (μg)	Reference to dietary survey.
Thiamin intake of 31 population groups of Northern India.					
1.	Five middle class families of Lahore.	1829	3500	0.52	Unpublished survey by the authors.
2.	Six well-to-do Hindu families.	1466	3200	0.46	Wilson and Widdowson (1942)
3.	Eight middle class Hindu families.	1327	2655	0.50	,,
4.	Six poor class Hindu families.	690	2100	0.33	,,
5.	Sixteen middle class Muslim families.	1125	2542	0.44	,,
6.	Twenty-two poor class Muslim families.	1032	2284	0.45	,,
7.	Twenty-three middle class Sikh families.	1434	2840	0.50	,,
8.	Fourteen poor class Sikh families.	1486	2227	0.54	,,
Rural wheat-eating communities of Punjab.					
9.	Nine middle class families.	1425	3042	0.47	,,
10.	Nine poor class families.	1319	2391	0.55	,,
Urban wheat-eating communities of Punjab.					
11.	Nine middle class families.	1480	2430	0.60	,,
12.	Nine poor class families.	1167	2342	0.50	,,
Different tribes of cultivators in the rural areas of Lyallpur District.					
13.	Fifteen families of Arains (A).	2041	3854	0.53	*Report Punjab Public Health Department* (1943).
14.	Fifteen families of Arains (B).	2070	4106	0.50	,,
15.	Fifteen families of Muslim Jats.	2346	4321	0.54	,,
16.	Fifteen families of Sikh Jats.	2346	5055	0.46	,,
17.	Fifteen families of Janglies.	2107	3822	0.55	,,

TABLE I.—(*Contd.*)

No.	Description of the population group.	Total thiamin intake on cooked food basis (μg)	Total calorie intake.	Thiamin intake per calorie. (μg)	Reference to dietary survey.
Different types of cultivators in the Lyallpur District.					
18.	Fifty families of owner-cultivators.	3037	4184	0.72	*Report Punjab Public Health Department (1943).*
19.	Seventeen families of owner-tenants.	2183	4290	0.51	,,
20.	Eight families of land-less tenants.	2244	4121	0.54	,,
21.	Average of above seventy-five families cultivators.	2124	4197	0.51	,,
Different centres in the Kangra Valley.					
22.	Fifteen families of Launa village.	436	2468	0.18	*Report Punjab Public Health Department (1939).*
23.	Fifteen families of Gorra village.	1062	3029	0.35	,,
24.	Fifteen families of Chilali village.	1487	2923	0.51	,,
25.	Fifteen families of Bhadwar village.	1717	3576	0.48	,,
Different centres of Kangra Valley at different seasons.					
26.	Sixty families of Launa, Gora, Chilali and Bhadwar in pre-monsoon period.	1221	3009	0.41	*Report Punjab Public Health Department (1939).*
27.	Sixty families of Launa, Gora, Chilali, and Bhadwar in Monsoon period.	1105	2939	0.38	*Report Punjab Public Health Department (1939).*
Individuals of different communities in the Kangra Valley.					
28.	A Brahmin.	1300	2975	0.44	*Report Punjab Public Health Department (1939).*
29.	A Chamar.	1393	2526	0.55	,,
30.	A Girth.	334	1964	0.17	,,
31.	A Weaver.	496	2819	0.18	,,

It is well recognised that calorie intake is of importance in considering the requirements of thiamin. A somewhat moderate intake of thiamin may be adequate when total calories intake is low and the build of the individual small, but it may prove totally inadequate with a large calorie intake and a somewhat bigger body build. In Nutrition surveys undertaken in this country it is rarely that calorie intake has been given sufficient consideration. While this point may not be of such great importance for urban population groups consuming moderate calories, in rural areas dealing with population groups of bigger body build and engaged in hard manual labour such as those of land cultivatore, consuming over 4,000 calories, this subject assumes considerable importance. It may also be pointed out that in dealing with a substance which is readily destroyed, with exposure to heat and when losses are large in methods of cooking employed, the calculation of dietary intake on the basis of raw foods, a practice generally followed in such investigations may give a totally erroneous idea of the state of things. For studies with respect to thiamin, the estimates of intake must therefore be made on the cooked food basis as has been done in this investigation.

In the above table the data on the total thiamin intake as well as the thiamin intake per calorie calculated for each populations group has been presented. It would naturally be of interest to examine these in the light of thiamin requirements. The standard requirements of thiamin, which have been the subject of considerable investigation and thought, by different authorities, are shown in Table II.

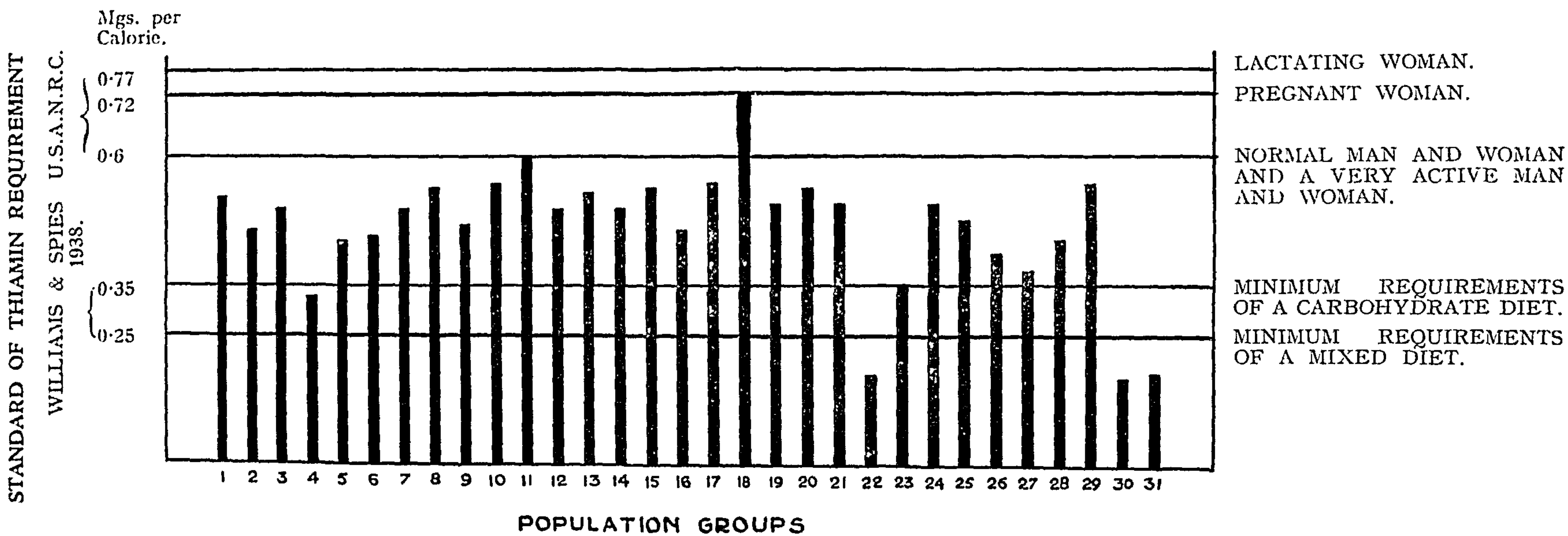
TABLE II.

Standard vitamin B_1 requirements.

Dietary condition.	Physiological state of the individual.	Standard of calories.	Standard total daily requirement of thiamin.	Standard requirement of thiamin per calorie.	Reference.
Mixed European diet	—	3,000	0.75 mg.	0.25 mg.	Williams and Spies (1938)
Non-fat carbohydrate diet	—	3,000	1.05	0.35	,,
Normal mixed diet	Normal man	3,000	1.80	0.60	Food and Nutrition Board, National Research Council U.S.A.
,,	Very active man	4,000	2.30	0.60	,,
,,	Normal woman	2,500	1.50	0.60	,,
,,	Very active woman	3,000	1.80	0.60	,,
,,	Pregnant woman	2,500	1.80	0.72	,,
,,	Lactating woman	3,000	2.30	0.77	,,

In the light of these standards, the position of the 31 diets is graphically shown in Fig 1. It will be noticed that all diets fall short of the standard for lactating woman, only one out of the 31 diets comes upto the standard for pregnant woman,

CHART SHOWING DAILY VITAMIN B_1 INTAKE OF DIFFERENT POPULATION GROUPS IN PUNJAB



and only two diets are upto standard for a normal man or woman or a very active man or woman according to the allowances recommended by the Food and Nutrition Board of the National Research Council, U.S.A. Williams and Spies have estimated the requirements for a mixed diet to be 0.25 μg per calorie and for a non-fat carbohydrate diet 0.35 μg per calorie. The diets which we have been considering are in no way comparable to a mixed European diet for which Williams and Spies have estimated the normal requirement of 0.25 μg per calorie. The diets used by these communities in Northern India are more comparable to a non-fat diet since they consist very largely of carbohydrate with only negligible amounts of fat. Therefore, according to this estimate the minimum requirement for these human groups would be 0.35 μg per calorie. Considering this as the minimum requirement we find that 4 diets are below the standard, while all the rest are adequate.

While considering the adequacy of these diets with respect to thiamin, climatic factors also need to be taken into consideration. It is well established that in fever and in hyperthyroidism there is an increased requirement of vitamin B_1. A very interesting observation has been made by Mills on the effect of external temperature on thiamin requirement. He has found that young rats maintained at an environmental temperature of 91° F. require double the amount of thiamin as compared to animals kept at 65° F. If this observation is correct and is applicable also to humans in tropical regions where environmental temperatures are high and range between 100-115° F. in summer, the requirement of vitamin B_1 must be higher than those commonly determined for temperate climates.

In view of these facts it can be concluded that many sections of the population in this region, contrary to common belief, are by no means receiving a diet containing normal allowances of thiamin as commonly recommended. The diets of some of the poorer sections of the population is inadequate in this factor. According to medical opinion signs which could be ascribed to thiamin hypovitaminosis are not uncommon and a large number of medical practitioners continue to prescribe thiamin therapy with considerable success in certain conditions of undefined aetiology. Further studies on this subject are in progress.

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VITAMIN 'A' DESTRUCTION IN SHARK LIVER OIL (ZYGAENA BLOCHI) AND THE EFFECT OF ADDING ANTIOXIDANTS

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Vitamin 'A' destruction in Shark liver oil.—Fresh body and liver oils have no strong flavour. However, they develop the characteristic disagreeable odour on storage. The development of bad odour is mainly due to oxidation. Peroxides are formed as a result of oxidation and they destroy the vitamin 'A' present in fish liver oils (1). Vitamin D is comparatively more stable than vitamin 'A'. The oxidation of vitamins by peroxides takes place more rapidly at higher temperatures and it has been observed that in a tropical country like India cod liver oil, halibut liver oil and other vitamin concentrates lose their vitamin potency on storage at a comparatively rapid rate (2). So the problem of preserving fish liver oils is one of great importance in this country.

Several workers have studied the stability of vitamin 'A' in fish liver oils under different conditions of storage (3, 4, 5). They have recorded wide variations in the amount of Vitamin 'A' destroyed (6, 7, 8, 9, 10, 11, 1). Since the destruction of vitamin 'A' in fish liver oils is mainly due to oxidation a convenient method of retarding oxidation changes is to add an antioxidant to the oil. Ever since 1922 when Moureu and Dufraisse reported the effectiveness of hydroquinone as an anti-oxidant a vast number of materials have been described in literature as possessing the property of inhibiting oxidation changes in oils and fats. Some of these are phenolic compounds as hydroquinone (12), pyrogallol (13) α-and β-Naphthol (14) ; numerous organic nitrogen compounds including various amines (15), hydrazines (16), diphenyl guanidine (17) cyanamide (18) ; several chlorinated and brominated paraffins (19) ; polybasic acids such as phthalic and maleic (12) and such materials as gum guaiac (20) Lecithin (21), and crushed oil bearing seeds and cereal flours (22). Many of these substances are toxic and so are use-

less for edible fats. Recently, an observation was made in these laboratories that Kamala dye not only colours ghee but also helps to preserve it (23).

In India a very valuable source of vitamin 'A' is shark liver oil. The preparation of shark liver oil rich in vitamin 'A' is now an established industry on the west coast of India. Information is not available about the factors which influence vitamin 'A' destruction in shark liver oil. The present investigation was undertaken with a view to obtain information on the subject and also to find out the effectiveness of antioxidants in retarding vitamin loss.

Materials and procedure.—The shark liver oil used for the experiments was kindly supplied by the Department of Fisheries, Government of Madras.

Under ordinary conditions of storage vitamin 'A' destruction takes place slowly in fish liver oils. To find out the effect of adding antioxidants and determine their efficiency within a reasonable length of time some kind of mild acceleration to promote oxidation changes is required. So thin layers of oil were exposed to the air at elevated temperatures.

During summer months the temperature in the plains of India often exceeds 40°C. and so it was decided to store the oil at that temperature and thus as far as possible follow conditions during usual methods of storage. 10 ml. samples of shark liver oil were stored in 100 ml. Erlenmeyer flasks fitted with loose stoppers allowing passage of air. Weighed quantities of the antioxidants were directly added to the oil. The flasks were kept in an electrically maintained incubator. No light was allowed to reach the samples during storage. The peroxide value and the vitamin 'A' content were determined at periodical intervals. Peroxide value was measured by the method described by Wheeler (24).

The vitamin 'A' content of the shark liver oil was measured in a Hilger Vitameter A, using chloroform as solvent. No attempt was made to first separate the unsaponifiable fraction before determining the vitamin 'A' content in the oil because it is known that when peroxides and vitamin 'A' are present in a system, the peroxides very rapidly oxidise vitamin 'A' at elevated temperatures (25) such as the one used for the saponification of the oil. An objection to the spectroscopic method may be taken in that the oxidised vitamin 'A' may also to a certain extent take part in absorption of ultraviolet light. The Carr-Price test does not offer a better method of determination because it is known that the oxidised cholesterol present in fish liver oil also gives a colour. It is true that the biological method is more reliable but as Lease *et al* (1) have shown the destruction of vitamin 'A' by peroxides would continue even during the course of the bio-assay. Hence the biological method is of limited usefulness in this case.

The results obtained after storing the oil at 40°C. in the dark are given in the following table. The oil was found to be very rich in vitamin 'A' and the factor for converting. $E_0 = \frac{1 \text{ cm.}}{1\%}$ 328 mμ. into international units was taken as 1100.

TABLE I
The Vitamin 'A' content and peroxide value of shark liver oil.

Time in days.	Peroxide value				Vitamin content in I.U.			
	Control.	Oil + 0.01% K.D.	Oil + 0.02% K.D.	Oil + 0.05% H.Q.	Control.	Oil + 0.01% K.D.	Oil + 0.02% K.D.	Oil + 0.05% H.Q.
0	Nil	Nil	Nil	Nil	9310	9310	9310	9310
7	Nil	Nil	Nil	Nil	9200	9320	9310	9310
14	10.1	8.2	7.2	Nil	8070	8775	8975	9055
21	84.2	45.4	38.7	7.1	6560	8030	9395	8600
28	210.6	101.1	84.6	15.2	4125	6000	6550	8135
35	237.8	232.1	223.5	80.4	1550	3610	4450	6605

The results are also represented graphically in Fig. 1. Peroxide formation in the untreated sample has taken place more rapidly than in the treated samples. Kamala dye and hydroquinone have both retarded the formation of peroxides but towards the end of the period of storage i.e., after about five weeks, the amount of peroxides in all the samples is more or less same. As will be mentioned below the vitamin contents of the different samples after five weeks storage are not the same indicating that the peroxide value is of limited usefulness, in following vitamin 'A' destruction in fish liver oils.

Vitamin destruction has taken place very rapidly in the unterated control, the vitamin content after five weeks of storage being barely one sixth that of the original sample. Even the addition of 0.1% Kamala dye has appreciably retarded vitamin loss while the results obtained after the addition of 0.2% of the dye are even better. Addition of 0.05% hydroquinone was very helpful in retarding the destruction of vitamin 'A' and it acted as a more efficient antioxidant than Kamala dye.

Effect of light.—Vitamin 'A' exhibits its characteristic absorption spectra near 328 mμ and according to the Grotthus Draper law one may expect that light of wavelength 328 mμ can produce photochemical changes in vitamin 'A'. These theoretical considerations have been confirmed in practice as a result of research work carried out in different parts of the world (26, 27, 28). Several workers have recorded that vitamin 'A' is destroyed by irradiation (29, 30). But Heilbron *et al* (31) also observed that in liver oils which with the antimony trichloride test showed a preponderance of the 572 mμ chromogen over that of the 606 mμ an increase in the strength of the 606 mμ occurred after several months storage together with a small increase in the strength of the extinction coefficient of the oils at 320 mμ (32, 33, 34).

In view of divergent results obtained by several workers it was thought desirable therefore to find out the action of light on Indian shark liver oil and also the protection afforded by anti-oxidants.

10 ml. samples of shark liver oil were stored in 100 ml. flasks at the laboratory temperature. The flasks were provided with loose fitting stoppers permitting

passage of air. One batch was stored in the dark. Another batch was kept on a window-sill facing north. The antioxidants were added to the oil directly. Diffused light passed through the glass panes and the walls of the flask before reaching the oil. No direct sunlight was allowed to act on the oil.

The samples were analysed for their vitamin 'A' content and peroxide value at frequent intervals. The following table shows the results obtained. The results are also graphically represented in Fig. 2 and 3. The laboratory temperature during the course of the experiments varied between 20°C. and 30°C.

TABLE II

Peroxide value of the samples in ml. and thiosulphate per Kg. of oil.

Time in days.	Samples stored in dark.			Samples stored in diffused light.		
	Control.	Oil + 0.1% K.D.	Oil + 0.2% K.D.	Control.	Oil + 0.1% K.D.	Oil + 0.2% K.D.
0	Nil	Nil	Nil	Nil	Nil	Nil
7	Nil	Nil	Nil	5.1	5.0	3.0
14	9.3	3.0	Nil	53.9	40.3	30.3
21	22.6	8.0	6.6	292.0	242.2	145.3
28	39.5	15.1	11.3	375.3	318.0	328.3
35	60.7	34.0	24.9	420.0	378.0	402.2

TABLE III

The vitamin content of the samples in International Units.

Time in days.	Samples stored in dark.			Samples stored in diffused light.		
	Control.	Oil + 0.1% K.D.	Oil + 0.2% K.D.	Control.	Oil + 0.1% K.D.	Oil + 0.2% K.D.
0	9310	9310	9310	9310	9310	9310
7	8898	9200	9256	8049	8870	9015
14	8870	9020	9310	7045	7960	8860
21	8304	9160	9390	5335	5325	5870
28	7989	8955	9165	2663	3065	3438
35	7895	8685	9115	2505	2750	3372

It will be seen from the tables that light has greatly accelerated peroxide formation. Addition of 0.1% Kamala dye retarded peroxide formation in the initial stages. 0.2% addition of the dye was slightly better but all the treated samples showed more or less the same peroxide content as the untreated one when examined after four weeks. Vitamin deterioration took place very quickly when the samples of the fish liver oil were exposed to diffused light. Addition of the antioxidant was helpful but the protective effect was not much. The samples stored in the dark showed higher vitamin content than those stored in diffused light.

A second batch of shark liver oil similarly prepared and kindly supplied by the Director of Fisheries, Government of Madras was used for these experiments. As before 10 ml. samples of the oil were stored in 100 ml. Erlenmeyer flasks fitted with loose stoppers allowing free passage of air. The temperature of the incubator was maintained at 40°C. The amount of vitamin 'A' in the samples was measured at frequent intervals in a Hilger Vitameter 'A'.

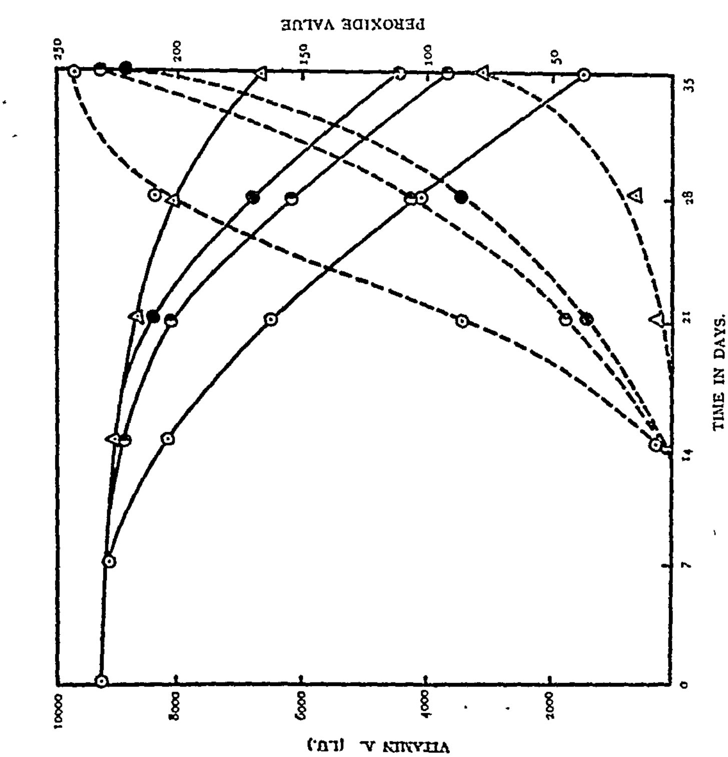

FIG. 1. THE VITAMIN A CONTENT AND PEROXIDE VALUE OF SHARK LIVER OIL STORED AT 40°C

VITAMIN A. CONTENT.	PEROXIDE VALUE.
CONTROL	CONTROL
+0.1% K.D.	+0.1% K.D.
+0.2% K.D.	+0.2% K.D.
+0.05% H.Q.	+0.05% H.Q.

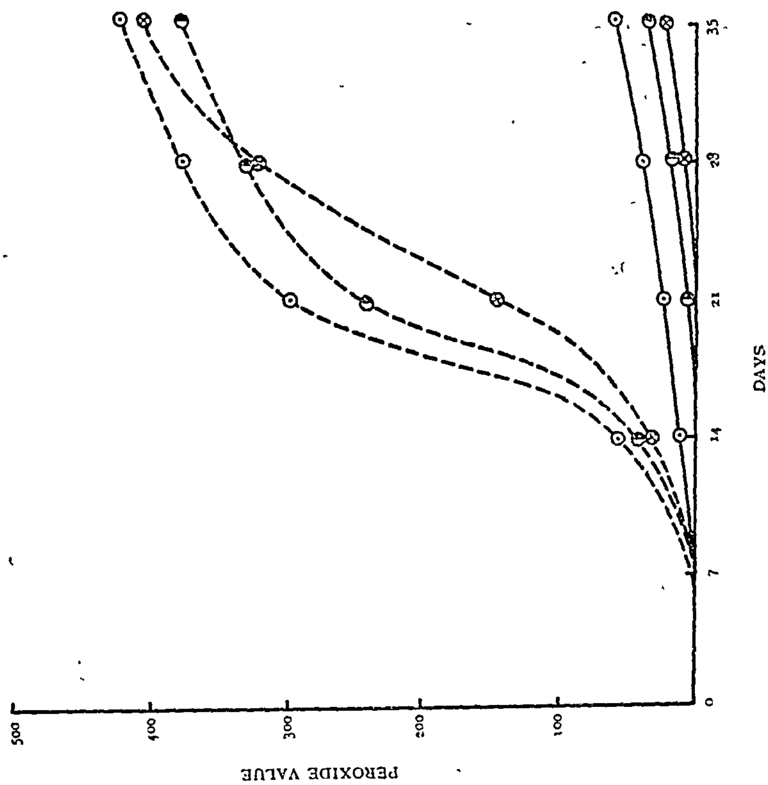

FIG. 2. PEROXIDE VALUE OF SHARK LIVER OIL STORED IN DIFFUSED LIGHT AND IN DARK

OIL EXPOSED TO DIFFUSED LIGHT	OIL KEPT IN DARK
CONTROL	CONTROL
OIL +0.1% K.D	OIL +0.1% K.D.
OIL +0.2% K.D.	OIL +0.2% K.D

The following antioxidants were used:—

(1) 0. 1% Kamala
(2) 0. 05% Kamala + 0.02% Maleic acid
(3) 0. 05% Hydroquinone
(4) 0.025% Hydroquinone + 0.05% Kamala dye
(5) 0.1% Kamala + 0.5% Oleic acid.

A number of combinations are possible but only a few have been selected. Weighed quantities of the antioxidants were directly added to the oil. The results obtained are presented in Table IV. The results are also graphically represented in Fig. 4.

TABLE IV

Synergetic effect of antioxidants.

Time in days.	Vitamin content of the samples in I U.					
	Blank.	Oil + K D	Oil + K.D + Maleic acid.	Oil + H.Q.	Oil + H.Q. + K.D.	Oil + K.D. + Oleic acid.
0	7310	7310	7310	7310	7310	7310
8	7150	7406	7461	7328	7267	7586
12	7031	7156	6924	7473	6734	6563
16	3434	6512	5980	5980	6122	5687
20	2995	3948	4143	5453	5909	5319
24	2495	3846	3774	5145	5545	5089

The results show that vitamin loss has been taken place very rapidly after ten days in the untreated sample. After about sixteen days the oil became very viscous. The high viscosity of the oil probably prevented the access of oxygen to the deeper layers of the oil. The retardation in the destruction of vitamin 'A' after sixteen days may be due to this cause viz., the high viscosity of the oil. Addition of 0.1% Kamala dye is very helpful in preventing vitamin loss. A combination of 0.05% Kamala dye and 0.02% maleic acid is as effective as 0.1% Kamala dye. In this case also the oil became very viscous after twenty days. The retardation in vitamin loss after this period may be due to the high viscosity of the oil. Hydroquinone is a powerful antioxidant and addition of even 0.05% hydroquinone greatly retarded vitamin loss. Because of the toxic nature of the inhibitor in high concentrations, hydroquinone may not be acceptable to the public. If instead of adding 0.05% hydroquinone, a mixture of 0.025% hydroquinone and 0.05% of less toxic Kamala dye is used, the effect is more or less the same. The synergetic effect of Kamala dye and oleic acid is remarkable. When oleic acid is added along with Kamala dye, the two act in combination and the inhibiting effect is greater than that of Kamala dye alone. From the tables it will be seen that the sample treated with Kamala dye has retained only about $\frac{1}{3}$ the original amount of vitamin 'A' after twenty four days. But in the case of the sample treated with Kamala dye and oleic acid, the sample retained more than $\frac{2}{3}$ the amount. This fact is interesting because oleic acid is an unsaturated acid and so it is by itself prone to oxidation changes. When present along with an antioxidant like Kamala dye the two act as an antioxidant combination.

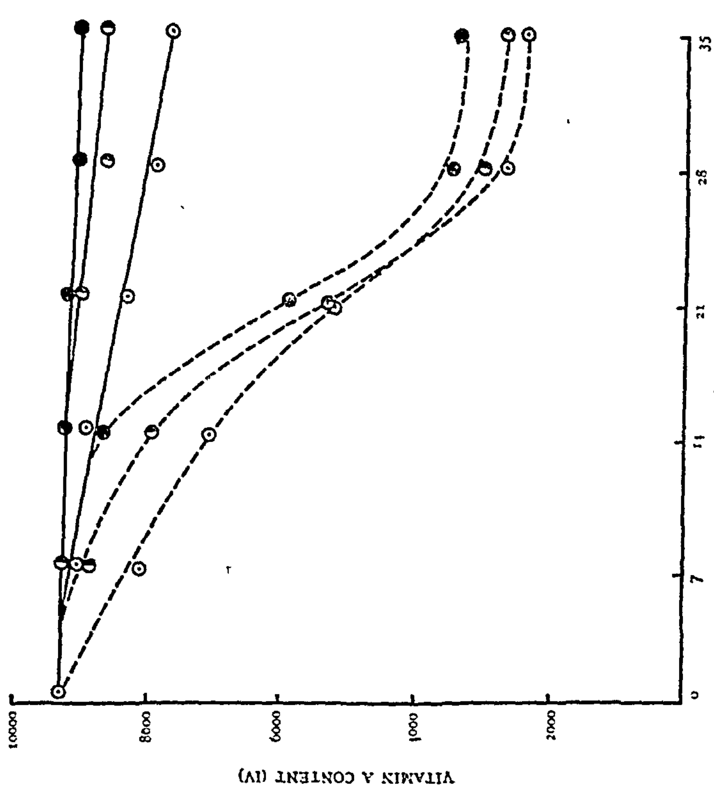

FIG. 3.

VITAMIN A CONTENT OF SHARK LIVER OIL STORED IN DIFFUSED LIGHT AND IN DARK

OIL EXPOSED TO DIFFUSED LIGHT

CONTROL

OIL +0.1% K.D.

OIL +0.2% K.D.

OIL KEPT IN DARK

CONTROL

OIL +0.1% K.D

OIL +0.2% K.D.

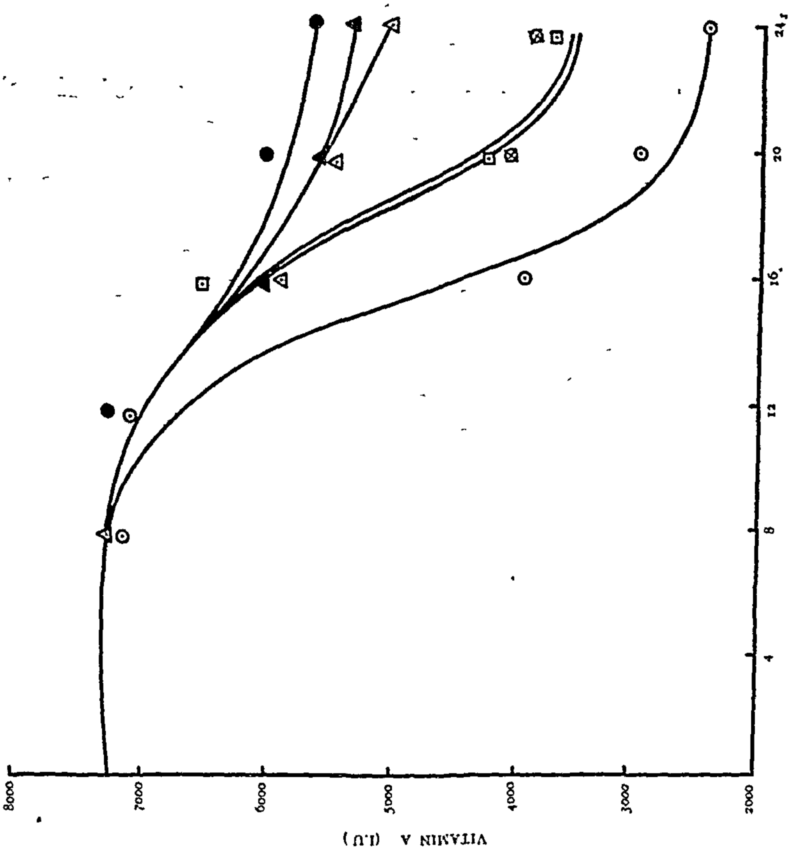

FIG. 4.

THE SYNERGETIC EFFECT OF ANTIOXIDENTS.

CONTROL

OIL +0.1% K.D.

OIL +0.05% K.D. +0.02% MALEIC ACID

OIL +0.1% K.D. +0.5% OLEIC ACID

OIL +0.05% HYDROQUINONE

0.1+0.5% K.D. +0.025 H.G.

Summary

(1) Loss of vitamin 'A' in shark liver oil on storage has been studied. Kamala dye and hydroquinone retarded vitamin 'A' loss in shark liver oil.

(2) On exposure to diffused light peroxide formation and vitamin destruction in shark liver oil took place more rapidly compared with samples stored in the dark. The protection afforded by the antioxidant was greater when the oils were stored in the dark than with the samples stored in diffused light.

(3) Mixtures of Kamala dye and maleic acid, Kamala dye and hydroquinone, Kamala dye and oleic acid retarded vitamin 'A' loss in shark liver oil better than the dye alone. Particularly when oleic acid was present the synergetic effect was remarkable.

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STORAGE OF GHEE: INFLUENCE OF THE METHOD OF PREPARATION AND ACIDITY ON THE KEEPING QUALITY OF GHEE

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The resistance of ghee towards rancidity and vitamin 'A' destruction by heat and oxygen has been attributed to the presence of different amounts of protective substances present in it but since ghee itself is prepared from butter, the method of preparing butter may profoundly influence the keeping quality of ghee prepared from it. Butter may be prepared from milk by two processes, viz., the curd process and the cream process. In the curd process, pasteurised whole milk is taken, inoculated with lactic acid culture, soured, and the curd obtained is churned to get butter. In the cream process, pasteurised milk is passed through a cream separator. The separated cream comparatively rich in fat, the fat content varying from 30% to 60%, is then inoculated with lactic acid culture. After souring the soured cream is churned and butter is prepared from it.

Previous work in these laboratories indicated that Kamala dye is a promising antioxidant for ghee (Govindarajan and Banerjee, 2). Unfortunately the experiments were conducted using commercial samples of ghee. The principal object of this investigation was to test the effectiveness of Kamala dye as an antioxidant on genuine fresh samples of ghee, prepared by both the curd and cream processes.

Fresh pasteurised cow's milk was taken and inoculated with a mixed culture of lactic acid bacteria. Immediately after the milk had coagulated the curd was churned and butter prepared out of it. Another portion of the milk was first passed through an 'Alfa Laval' cream separator. The cream obtained was inoculated with a mixed culture of lactic acid organisms. After ripening, the cream was churned, and butter prepared. The two samples of butter were washed well and immediately melted to prepare ghee. When the separated fat attained a temperature of 105°C. heating was stopped. The final products obtained possessed good ghee aroma. The samples of ghee so obtained were used for the experiments. The acidity of the ghee samples was determined.

The acidity of ghee prepared by the cream process was 0.06% and that of the curd process ghee was 0.4% expressed as oleic acid.

The onset of rancidity usually coincides with or follows shortly after the end of the induction period. So to get an idea about the keeping quality of the various samples of ghee and also to test the efficiency of the antioxidant, the method of determining the induction period and the amount of oxygen absorbed described by French, Olcott and Mattill (2) was adopted with suitable modifications. Erlenmeyer flasks containing the fats to be tested were immersed in an electrically maintained constant temperature oil bath. Each flask was fitted with a rubber stopper carrying an inlet and an outlet tube for oxygen both fitted with stopcocks and also an open mercury manometer to denote changes in pressure. Dry oxygen was passed through the flasks for about ten minutes and then the stopcocks on both the inlet and the outlet tubes were closed. Any pressure difference that might have been set up was equalized by momentarily opening the outlet stopcock. The reading of the two levels of the mercury manometer were taken at convenient intervals after absorption had started. The pressure changes due to temperature variation in the oil bath rarely amounted to 1 mm. and hence the absorption of even 0.5 ml. of oxygen resulted in a noticeable fall in pressure. The time of the beginning of oxygen absorption was taken to commence when a difference in levels in the mercury manometer was observed. Slight changes in temperature and atmospheric pressure as measured by pressure changes in a flask containing no fat in the course of the experiments were not appreciable and were neglected. The temperature of the bath was maintained at 95°C. and 20 grams of fat was taken for each experiment. Weighed quantities of the antioxidants were directly added to the fat.

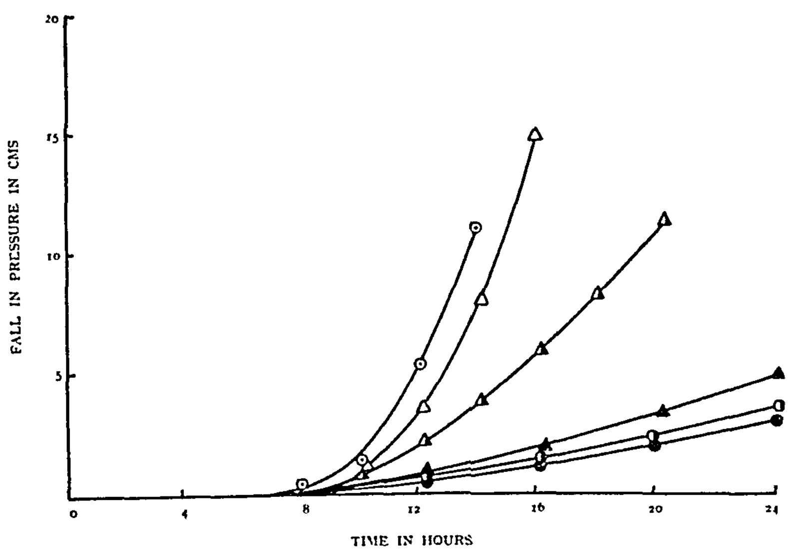


FIG A OXYGEN ABSORPTION OF GHEE

CURD PROCESS CONTROL
" +0·02% K D
" +0·05% K D
CREAM PROCESS CONTROL
" +0·02% K D
" +0·05% K D

Table I presents the results obtained. The oxygen absorption is represented graphically in Fig. A.

TABLE I

Substrate.	Inhibitor added.	Induction period.	Antioxygenic index.
1. Ghee (cream process)	Nil	6 hours	...
,, ,,	+ 0.02% Kamala dye	6 hours	1
,, ,,	+ 0.05% ,,	6 hours	1
2. Ghee (curd process)	Nil	7 hours	...
,, ,,	+ 0.02% Kamala dye	7 hours	1
,, ,,	+ 0.05% ,,	7 hours	1

The antioxygenic index is the ratio of the induction period of the untreated sample to that of the treated sample. It will be observed that the antioxidant does not appreciably prolong the induction period of the ghee samples. Ghee samples prepared both by the cream process and the curd process do not differ much as regards their keeping quality. However, the rate of oxygen absorption after once the induction period was over was very much reduced by the addition of the antioxidant. The protection afforded by the antioxidant was not strictly proportional to the amount used. The effectiveness of the antioxidant was more marked in the case of ghee prepared by the curd or 'deshi' process. The greater protection afforded by the antioxidant in the case of curd process ghee may be due to the synergetic effect of Kamala dye, along with some other constituent present in ghee, possibly the free fatty acids. The results bring out that another aspect of the problem should be given due consideration i.e., the effect of acidity.

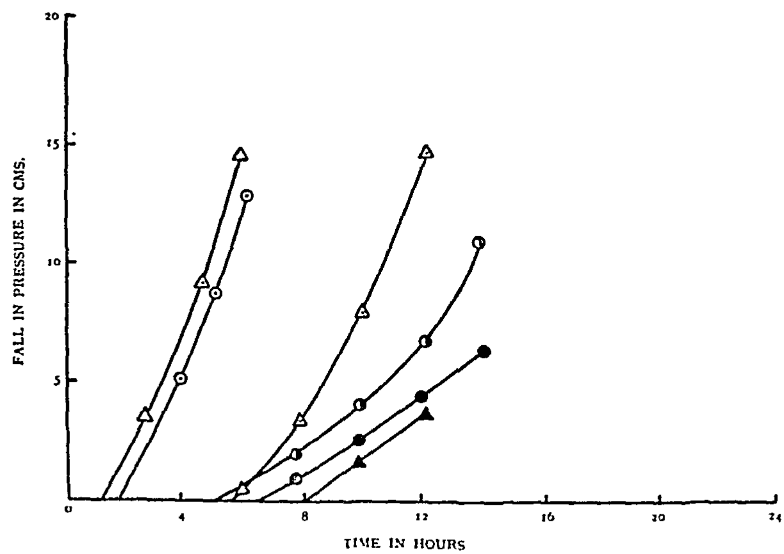
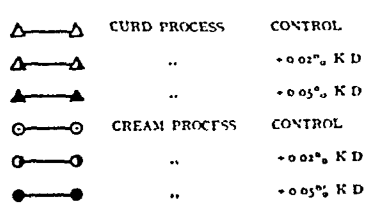


FIG B OXYGEN ABSORBTION OF ACIDIFIED GHEE



Effect of acidity.—In India the chief centres of production of ghee are villages and the universal method adopted for preparing the butter is the curd process. Lactic fermentation of milk takes place very quickly in the summer season when the thermometer often registers a temperature over 100°F. Because of the want of proper control over the process of manufacture the acidity of commercial samples of ghee prepared by the curd process usually varies from 1% to 5% expressed as oleic acid.

To find the effect of acidity the sample of curd and cream process ghee used in the previous experiment were taken and sufficient oleic acid was added to raise the acidity to 2%. The induction period and the oxygen absorption of the samples were determined. The results are given in the following table and graphically in figure B.

TABLE II

Effect of acidity.

Substrate.	Inhibitor added.	Induction period.	Antioxygenic index.
1. Ghee (curd process)	Nil	1.5 hours	...
,, ,,	+ 0.02% Kamala dye	5.5 hours	3.6
,, ,,	+ 0.05% ,,	8.0 hours	5.3
2. Ghee (cream process)	Nil	2 hours	...
,, ,,	+ 0.02% Kamala dye	4.6 hours	2.3
,, ,,	+ 0.05% ,,	6.2 hours	3.1

It will be obsevered that acidity plays a great part in determining the length of the induction period. The induction period of the two samples were respectively 1.5 hrs. and 2 hrs. This more or less corresponds to the 2 hrs. induction period for the commercial sample of ghee used in the previous paper. Oleic acid shortened the length of the induction period but when Kamala dye was present the two together acted as an antioxidant combination. So the beneficial effect of Kamala dye on commercial samples of ghee must be very likely due to the synergetic effect of the antioxidant and the free acid present in the samples of ghee. Since commercial samples of ghee usually have got a high acidity the use of Kamala dye as an antioxidant for such samples has got much in its favour.

Summary

1. Butter was prepared by cream process and curd process. The induction period of ghee obtained from the butter samples was determined. It was found that the induction period of ghee was not much influenced by the method of preparation of butter, so long as the acidity was low. The protection afforded by Kamala dye on low acidity ghee samples was not much.

2. The ghee samples were acidified with oleic acid. Addition of acid greatly reduced the induction period.

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ROLE OF VITAMINS IN THE METABOLISM OF CALCIUM, MAGNESIUM AND PHOSPHORUS IN HUMAN SUBJECTS

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With progress of research in the field of nutrition some evidence has accumulated to show that the vitamins possess some beneficial effect on the utilisation of nutritionally essential elements as calcium, magnesium and phosphorus. A brief study of the different work in this line is discussed below.

Vitamin A: Literature regarding the role of vitamin A on the metabolism of essential elements is meagre. Bogert and Trail (1) and Macy *et al* (2) found improvement in the balance of calcium, magnesium and phosphorus after administering butter fat to adult women.

Vitamin B: Knowledge regarding the role of Vitamin B complex on the metabolism of calcium, magnesium and phosphorus is firmly restricted to the work of Macy *et al* (2), who found that the addition of yeast to the diet of adult women improved the retention of the above elements.

Vitamin C: Investigations of Holst and Frolich (3) Howard and Ingvaldsen (4); Howe (5) Robb *et al* (6) ; Toverud (7) ; Mellanby (8) and Michaux (9) do not show any clear relationship between scurvy due to vitamin C deficiency and the metabolism of calcium and phosphorus. Although Chany and Blunt (10, 11) found increased retention of calcium, magnesium and phosphorus and nitrogen following orange juice ingestion and attributed the increase to the vitamin C content of the juice, Daniels and Everson (12) did not find any improvement in the retention of the above elements when pure ascorbic acid was ingested.

Vitamin D: Though vitamin D helps in the utilisation of calcium and phosphorus, its function in utilisation of magnesium is a question of great controversy.

From the above discussion it becomes clear that there are conflicting views regarding the role of different vitamins in the utilisation of calcium, magnesium and phosphorus. The purpose of the present investigations was, therefore, to obtain a clear cut picture of the roles of different vitamins, in the metabolism of the above elements.

Most of the work discussed above was carried out on rats, chicks, etc. and a few were carried on human subject.

In the present investigation all work was carried out by direct metabolic experiment on adult human subjects.

Experimental

Experimental procedure and the methods were the same as those are employed in previous investigations reported from this laboratory by Basu and Basak (13) ; Basu, Basak and Rai Sircar (14) ; Basu, Basak and De (15) and Basu, De and Basak (16). The supplement of different vitamins was given after a "collection" period of 3 days when the subject was on the basal diet and the supplementation was continued for a further period of six days including two periods of 3 days. The composition of the basal diets (rice-fish diet and whole-wheat diet) used in the present investigation is indicated in Table I (a). In most cases urine and faeces were collected for six consecutive days during which supplements were given. Each period consisted of three consecutive days and the arithmetical numbers indicate the chronological order of the experimental periods. Period I was the preliminary period and does not figure in the tables.

The role of the following vitamins in the utilisation of calcium, magnesium and phosphorus has been studied by direct metabolic experiments on 4 human subjects (adult males) and the data are presented in Tables II to VIII.

Table I (b) represents the data regarding the content of different vitamin of the basal diets D I and D II.

Vitamin A—(Pure carotene and "Prepalin"—Glaxo product).
Vitamin B—complex (Yeast concentrate—("marmite).
Vitamin C—(Pure Ascorbic acid).
Vitamin D—(Irradiated ergosterol—"Vigantol," Merck-Bayer).
Vitamin B_1—(aneurin—"Benerva" Roche).
Heat stable factor of Vitamin B-complex (Autoclaved marmite).
Riboflavin.

Methods of Analysis

Following methods were adopted for estimation of Calcium, Magnesium and Phosphorus of basal diets, urine and faeces.

Calcium and Phosphorus—Hawk and Bergeim,.

Magnesium—Greenberg and Mackey (17).

TABLE I (*a*)

Composition of the diets expressed in grammes.

	Non-vegetarian rice diet (D I).	Whole wheat diet (D II).
Rice	600	550—600
Pulse	60	100
Fish*	70	...
Vegetables:		
Potato	100	100
Brinjal, Bean, Potol, Pumpkin gourd	100	100
Butter fat	...	30
Mustard oil	30	...

*Fish used was small fish as kakchi (*Conica Sobona*), Chapila (*Clupea Chapra*) and Mala (*Amblyphyaryngodon mola*) which supplied large amount of utilisable calcium to the poor rice diet [Basu, De and Basak (1942)].

TABLE I (*b*)

Vitamin contents of the diets.

Vitamin A and Carotene in International units	Range	680 —820
Ascorbic Acid in mg.	,,	15 — 18
Nicotinic Acid in mg.	,,	23 — 29
Vitamin B_1 (Aneurin) in mg.	,,	1.7 — 2.4
Riboflavin in mg.	,,	0.82— 1.64

Vitamin A Carotene.—The vitamin A and Carotene content of the basal diets was much below the standard—the content ranged from 680 to 820 international units. The adult requirement ranges from 3500 to 4500 international units per day.

Ascorbic acid.—The ascorbic acid content of the daily basal diet was also very low being 15 to 18 mg. The minimum requirement of an adult is from 25 to 30 mg. per day.

Nicotinic acid.—The dietary content of nicotinic acid was between 23 to 39 mg. and this value was slightly higher than the adult requirement of 15 to 20 mg. per day.

Vitamin B_1.—The basal diets were adequate with regard to the content of vitamin B_1 which ranged from 1.7 to 2.4 mg. per day. The adult requirement of this factor has been found to be about 1 mg.

Riboflavin.—The basal diets were just adequate with regard to the Riboflavin content. The adult requirement is 0.5 mg. per 1000 calories.

TABLE II

Showing the role of vitamin A in metabolism.

The figures are expressed in mg. per day.

	Experimental subject.	G. C. N.		H. P. D.	
	Diet.	D I	D II + 1000 units of Vitamin A	D I	D I + 1000 units of Vitamin A
Mineral.	Period.	P II	Av. of P III and P IV.	P II	Av. of P III and P IV.
Calcium	Intake	441.0	441.0	542.0	542.0
	Urine	131.1	96.6	101.3	86.4
	Feces	283.2	200.0	316.4	264.1
	Balance	+ 26.7	+ 144.4	+ 124.3	+ 191.5
Magnesium	Intake	431	431	498	498
	Urine	49.4	38.2	84.2	78.2
	Feces	244.7	190.0	298.1	261.0
	Balance	+ 138.9	+ 202.8	+ 115.7	+ 158.8
Phosphorus	Intake	1103	1103	1515	1515
	Urine	442	430	671	621
	Feces	502	444	692	593
	Balance	+ 195	+ 229	+ 152	+ 301

Vitamin A and Carotene contents of basal diets:
For subject G.C.N.—780 Units (International) per day.
For subject H.P.D.—710 Units (International) per day.

TABLE III

Showing the role of vitamin B-complex (Marmite-yeast product) in metabolism.

The figures are expressed in mg. per day.

	Experimental subject.	G. C. N.		G. C. D.	
	Diet.	D II	D II + 10 g. Marmite (Yeast product).	D I	D I + 10 g. Marmite (Yeast product).
Mineral.	Period.	P II	Av. of P III and P IV.	P II	Av. of P III and P IV.
Calcium	Intake	484	484 + 3* = 515	615	615 + 31* = 646
	Urine	102.1	88.4	98.7	72.4
	Feces	284.5	262.3	398.9	353.2
	Balance	+ 97.4	+ 164.3	+ 117.4	+ 220.4
Magnesium	Intake	341	341 + 46* = 387	429	429 + 46* = 475
	Urine	45.3	40.2	84.2	74.2
	Feces	142.1	108.3	214.8	208.2
	Balance	+ 153.6	+ 238.5	+ 130.0	+ 192.6
Phosphorus	Intake	1132	1132 + 340* = 1472	1319	1319 + 340* = 1659
	Urine	342	302	612	541
	Feces	682	592	414	402
	Balance	+ 108	+ 578	+ 293	+ 716

* The extra calcium, magnesium and phosphorus in periods III and IV came from the marmite supplementation.

TABLE IV

Showing the role of vitamin B_1 in metabolism.

The figures are expressed in mg. per day.

Mineral.*	Experimental subject.	G. C. N.		G. C. D.	
	Diet.	D I	D II + 6 mg. Aneurin.	D I	D II + 6 mg. Aneurin.
	Period.	P II	Av. of P III and P IV.	P II	Av. of P III and P IV
Calcium	Intake	402	402	478	478
	Urine	23.4	14.6	66.8	86.2
	Feces	211.0	217.1	283.9	246.0
	Balance	+167.6	+170.3	+127.3	+145.8
Magnesium	Intake	261	261	416	416
	Urine	28.8	41.6	53.3	44.7
	Feces	97.7	79.4	292.4	289.3
	Balance	+134.5	+140.0	+70.3	+82.0
Phosphorus	Intake	1428	1428	1053	1058
	Urine	542	557	438	456
	Feces	673	650	512	482
	Balance	+213	+221	+103	+120

Vitamin B_1 content of basal diets:

For subjects G.C.N.:—1.9 mg. per day. For subjects G.C.D.:—2.1 mg. per day.

TABLE V

Showing the role of Heat stable factor of B complex (Autoclaved marmite—Yeast product) in metabolism.

The figures are expressed in mg. per day.

Mineral.	Experimental subject.	K. R. G.		G. C. N.	
	Diet.	D II	D II + 10 g. autoclaved marmite.	D I	D I + 10 g. autoclaved marmite.
	Period.	P II	Av. of P III and P IV.	P II	Av. of P III and P IV.
Calcium	Intake	462	462+31*=493	518	518+31*=549
	Urine	86.3	99.2	38.4	31.8
	Feces	278.5	216.4	343.4	314.2
	Balance	+97.2	+177.4	+136.2	+203.0
Magnesium	Intake	321	321+46*=367	351	351+46*=397
	Urine	35.4	37.4	98.4	78.2
	Feces	170.1	120.8	205.4	183.9
	Balance	+115.5	+208.8	+47.2	+134.9
Phosphorus	Intake	1148	1148+340*=1488	1594	1594+340*=1934
	Urine	438	421	498	482
	Feces	549	499	573	549
	Balance	+161	+568	+523	+903

* The extra calcium, magnesium and phosphorus in periods P III and P IV came from marmite supplementation.

TABLE VI

Showing the role of riboflavin in metabolism.

The figures are expressed in mg. per day.

Mineral.	Experimental subject.	K. R. C.		G. C. N.	
	Diet.	D I	D I + 20 mg. Riboflavin.	D II	D II + 20 mg. Riboflavin.
	Period.	P II	Av. of P III and P IV.	P II	Av. of P III and P IV.
Calcium	Intake	852	852	488	488
	Urine	175.0	119.8	48.8	34.2
	Feces	377.6	354.6	273.8	241.1
	Balance	+299.4	+377.6	+165.4	+212.7
Magnesium	Intake	421	421	360	360
	Urine	86.6	76.6	88.4	61.9
	Feces	242.6	135.4	182.1	141.5
	Balance	+91.8	+209.0	+89.5	+156.6
Phosphorus	Intake	1674	1674	1148	1148
	Urine	502	438	469	401
	Feces	702	641	563	516
	Balance	+470	+595	+116	+231

The riboflavin content of the basal diets:
For subject K.R.G.—1.27 mg. per day.
For subject G.C.N.—1.58 mg. per day.

TABLE VII

The role of vitamin C in metabolism.

The figures are expressed in mg. per day.

Mineral.	Experimental subject.	H. P. D.		K. R. G.	
	Diet.	D I	D I + 50 mg. Ascorbic acid.	D II	D II + 50 mg. Ascorbic acid.
	Period.	P II	Av. of P III and P IV.	P II	Av. of P III and P IV.
Calcium	Intake	836	836	581	581
	Urine	101.4	84.0	69.4	86.8
	Feces	520.1	326.5	401.4	312.8
	Balance	+214.5	+425.5	+110.2	+181.4
Magnesium	Intake	362	362	348	348
	Urine	46.9	45.8	64.8	68.8
	Feces	155.0	127.0	194.8	143.2
	Balance	+160.1	+189.2	+88.4	+136.0
Phosphorus	Intake	1865	1865	1494	1494
	Urine	690	592	502	532
	Feces	685	632	642	552
	Balance	+490	+641	+350	+410

Vitamin C content of the basal diet:
For subject H.P.D.—15 mg. per day.
For subject K.R.G.—17 mg. per day.

TABLE VIII

Showing the role of vitamin D in the metabolism.

The figures are expressed in mg. per day.

	Experimental subject.	G. C. N.		H. P. D.	
	Diet.	D I	D I + 1000 units of Vitamin D.	D I	D I + 1000 units of Vitamin D.
Mineral.	Period.	P II	Av. of P III and P IV.	P II	Av. of P III and P IV.
Calcium	Intake	513	513	617	617
	Urine	79.4	84.4	92.4	84.2
	Feces	389.3	302.5	458.4	401.9
	Balance	+44.3	+126.1	+66.2	+130.9
Magnesium	Intake	408	408	521	521
	Urine	74.2	59.3	106.8	84.2
	Feces	201.1	153.4	309.4	248.1
	Balance	+132.7	+195.0	+104.8	+188.7
Phosphorus	Intake	1421	1421	1618	1618
	Urine	568	556	582	561
	Feces	632	502	782	654
	Balance	+221	+363	+254	+403

RESULTS

Vitamin A: Data in table II indicate that the administration of 1000 units of vitamin A per day with the basal diets (vitamin A and carotene contents being 710 and 780 International units for subjects H.P.D. and G.C.N. respectively) increases the retention of calcium, phosphorus and magnesium to an appreciable extent. This observation supports the orginal work of Bogert and Trail (*loc. cit.*) and points out that the improvement in the retention of calcium, magnesium and phosphorus due to the administration of butter fat to the diet as observed by them was due to the presence of vitamin A in the butter fat.

Vitamin B-complex: Table III shows that vitamin B-complex ingested in the form of yeast concentrate (10 g. Marmite per day) enhances the utilisation of calcium, phosphorus and magnesium and so this vitamin B-complex seems to have a beneficial effect on the utilisation of the above minerals and the present results corroborate those of Macy *et al* (*loc. cit.*). Although marmite supplied moderate amounts of calcium, magnesium and phosphorus to the basal diet, its beneficial effect on the utilisation of above elements seems to be also quite distinct.

It was necessary then to study which factor of the vitamin B-complex was responsible for improving the utilisation of the above elements. The study of the heat labile and heat stable factors of the Vitamin B-complex was therefore, undertaken.

Heat labile factor of vitamin B-Complex

Vitamin B_1: Administration of 6 mg. of vitamin B_1—'aneurin' with the basal diets vitamin B_1 content being 1.9 and 2.1 mg. for subjects G.C.N. and G.C.D. respectively) decreased the elimination of calcium, magnesium and phosphorus to a slight extent (Table IV), and it may tentatively be concluded that vitamin B_1 has not got any appreciable effect on the metabolism of calcium, magnesium and phosphorous.

Heat-stable factors of vitamin B-complex

Administration of 10g. marmite, after destroying the heat labile factor of B-complex by autoclaving, shows a good effect on the economy of calcium, magnesium and phosphorus (Table V) and it seems that the beneficial effect of vitamin B-complex on the utilisation of essential elements as shown by the administration of unheated marmite (Table III) is not attributable to vitamin B_1 but to the heat-stable factors of the complex.

Riboflavin: Administration of 20 mg. of Riboflavin (Table VI) per day decreases the elimination of calcium, magnesium and phosphorus and therefore, it seems that riboflavin has got some beneficial effect on the utilisation of calcium, phosphorus and magnesium.

Since the other heat stable factors of B-complex were not available ready in hand it was not possible to carry out investigations with them.

Vitamin C: It will be evident from Table VII that the administration of 50 mg. ascorbic acid per day with the basal diets (ascorbic acid content being 15 and 17 mg. for subjects H.P.D. and K.R.G. respectively) greatly increased the retention of calcium, magnesium and phosphorus. The data presented are quite in accordance with those of Chaney and Blunt (loc. cit.) who observed better retention of calcium, magnesium and phosphorus due to administration of orange juice which supplied sufficient quantity of vitamin C to the diet.

Vitamin D: The results of the effect of vitamin D in the form of "vigantol" (Table VIII) show that the intake of 1000 units of vitamin D per day with the basal diets brought about better utilisation of calcium, phosphorus and magnesium by reducing their excretion.

Discussion

From the results it seems that vitamin A, B-complex, heat-stable factor of the B-complex, riboflavin, C and D help in the utilisation of calcium, phosphorus, magnesium. The beneficial effect of the vitamin B-complex in the utilisation of the above elements may either be attributed to the presence of riboflavin alone or also to some other heat-stable factor of B-complex.

From the survey of the results shown in the tables it will be observed that under the influence of vitamin A, B-complex, heat stable factor of the B complex, roboflavin, C and D the excretion of calcium, phosphorus and magnesium through feces in most cases decreased to a significant extent and this shows that the above vitamins facilitate the absorption of calcium, magnesium and phosphorus through the intestinal wall to appreciable extent.

The action of vitamin D in regulating the utilisation of calcium and phosphorus has long been known although the mechanism of its action is not yet clear. The beneficial effect of this vitamin in the utilisation of magnesium seems to be due to the improvement in the utilisation of calcium and phosphorus under the influence of this vitamin.

The increased assimilation of calcium and phosphorus under the influence of vitamin D may perhaps be due to increased acidity of the gut due to this vitamin and this increased acidity perhaps retards the precipitation of calcium phosphate and the removal of soluble ions like magnesium with the insoluble calcium phosphate. The individual precipitation of magnesium phosphates is also retarded resulting in better absorption of these elements through the intestinal wall.

The influence of other vitamins possessing beneficial effect on the utilisation of calcium, magnesium and phosphorus, may be concerned with some mechanism by which the precipitation of calcium and magnesium phosphates in the intestine is retarded with the net result of improved absorption.

SUMMARY

1. Vitamins A, B complex, C, D, the heat-stable factors of the vitamin B-complex and riboflavin help in the utilisation of calcium, phosphorus and magnesium.

2. The vitamin B_1 does not seem to make any significant improvement in the utilisation of the elements studied.

3. The beneficial effect of the vitamin B-complex on the utilisation of the above elements may be attributed to the presence either of riboflavin alone or also to some other heat stable factors of B-complex.

4. The improved utilisation of magnesium along with calcium and phosphorus under the influence of vitamin D has been explained on the basis of increased acidity of the gut and retardation of the precipitation of calcium and magnesium phosphate due to this vitamin with the net result of better absorption of the above elements through the intestinal wall.

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A COMPARATIVE STUDY OF BUTTER FAT AND HYDROGENATED VEGETABLE FAT (DALDA) ON THE UTILIZATION OF CALCIUM AND PHOSPHORUS

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A certain amount of fat in the diet is necessary for the solubility of calcium and phosphorous in the intestine. It is considered that there exists a competition in the intestine between phosphates and carbonates on the one hand and fatty acids on the other for combination with calcium. When too much phosphates is present in relation to fatty acids, insoluble calcium phosphate is formed and the calcium involved is lost to the body. Fatty acids combine with calcium to form calcium soaps which are then dissolved by bile salts to form water soluble diffusible complexes. Excess of fat, however, leads to a greater loss of calcium soaps in the stools and thus may interfere with calcium absorption and retention (Cronheim and Mueller, (1) ; French and Elliott, (2). Givens (3) observed that when fatty acid utilization is low, as is the case with ethyl palmitate or palmitic acid, the loss of calcium soap is increased.

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Bosworth, Bowditch and Giblin (4) concluded that the presence of soluble ionised calcium in the intestine determines the extent of soap formation, while loss in the feces depends not only upon the amount of calcium soaps formed but also upon their nature, calcium oleate being much more soluble in normal intestinal fluids than is calcium palmitate or calcium sterate. Boyd, Crum and Lyman (5) who studied the degree of absorption of various calcium soaps, found their utilization values in the following order: Calcium oleate 90%, Calcium palmitate 38%, and calcium sterate 25% when the calcium intake is 37 to 56 mg. per rat per day.

It would thus appear that the beneficial influence of different dietary fats in regaid to their action on calcium utilization will depend much on their chemical character and a study of the behaviour of hydrogenated vegetable fats in this respect is important. The process of hydrogenation of fats and oils introduces many points of interest for biochemical investigation.

Natural oils subjected to a process of hydrogenation are materially changed in the character of their fatty acids. Unsaturated acids are converted into solid saturated acids. Relative proportion of solid saturated acids of high molecular weight are increased. A new solid unsaturated acid called iso-oleic acid is formed.

The processing of vegetable oils by hydrogenation into edible products has now developed into a big and organised industry. The recent increase in their consumption is truely enormous. Large quantities of hydrogenated fats are used for adulteration of pure ghee and butter by unscrupulous merchants.

It has become now a common practice in many Indian households to use these vegetable products for cooking and other dietetic purposes in place of ghee or other vegetable oils. Being much cheaper in price, they are being increasingly in use even in those areas where milk fat could be had in abundance and at a reasonable price.

The question of digestibility of hydrogenated fats has received much attention. Researches on the nutritive properties of these products as compared to butter fat have resulted in conflicting conclusions. Recent work on infants (Holt and his coworkers, (6) indicates that the absorption of fat is favoured by the presence of fatty acids with one or two unsaturated linkages and of fatty acids with relatively short chains. In the present investigation, comparison has been made of the relative efficiency of butter fat and hydrogenated vegetable fat on the utilization of calcium and phosphorous in young rats.

Experimental Procedure

The absorption and retention of calcium and phosphorous has been measured by estimating the intake and output of these elements in urine and feces. Ten young healthy male albino rats with an average initial body weight of 48 gm. were selected for the test. All the rats were fed on a fat-free basal ration in the beginning and the urine and feces were collected for 10 days. The rats were then divided into two groups, the first group being given a diet containing 15% butter fat and the second, a similar diet containing 15% hydrogenated vegetable

fat. The collection of urine and feces was continued for another 10 days. Calcium was supplied in the diet as calcium lactate and was added at 3% of the ration. The composition of the diets is shown in table I.

TABLE I

Showing the composition of the diets.

Ingredients.	Fat-free diet.	Diet containing fats.
1. Casein (either extracted) ..	18	18
2. Rice starch	39	31
3. Sucrose	30	23
4. Yeast (ether extracted) ...	7	7
5. Calcium-free salt mixture (Osborne and Mendel No. 185, 1913)	3	3
6. Calcium lactate	3	3
7. Fat-Butter fat or Hydrogenated vegetable fat (Dalda)	—	15
	100	100

Each rat was given, in addition, 2 drops of Cod liver oil per day.

The percentage of Calcium and Phosphorous in the diets is shown in table II.

TABLE II

Showing the percentage of calcium and phosphorous in the diets.

Type of diet.	% Ca. in g.	% P. in g.
Fat free diet	0.552	0.635
Butter fat diet	0.50	0.670
Hydrogenated vegetable fat diet	0.511	0.660

Collection of Urine and Feces.—The rats were placed in individually metabolic cages and the urine was collected in bottles kept underneath the funnels over which the cages were placed. The feces was retained on a perforated porcelain disc which was kept within the funnel. Toluene and dilute hydrochloric acid were added to the collecting bottles of urine. The feces was removed daily and the funnels rinsed down with water. An accurate record of the food intake and gain in weight of the rats was kept during the experimental period. Distilled water was given ad-lib. for drinking purposes.

Methods of Estimation.—Calcium in food, urine and feces was determined by the Mc Crudden's method (7) in ashed material. Phosphorous was estimated by the colorimetric method of Bell and Doisy as modified by Briggs (8).

RESULTS

Food consumption and gain in weight of the rats is shown in table III.

TABLE III

Showing average food consumption, gain in weight and weight of dried feces.

Diet.	Number of rats used.	Initial body wt. g.	Final body wt. g.	Average daily food intake g.	Average gain in wt per day. g.	Average wt. of dried fæces in 10 days. g.
Fat-free diet	10	54	79.2	8.02	2.52	1.922
Butter fat diet	5	80.8	104	7.44	2.30	2.111
Hydrogenated fat diet	5	81.6	101.6	7.06	2.0	2.657

It will be seen that net food intake paralleled the growth. The faecal matter excreted by rats receiving hydrogenated fat diet weighed more than that of rats receiving butter fat diet. The weight of dried faeces on fat free diet was in general much less than on diets containing fats.

The results of calcium and phosphorous balance studies are shown in tables IV and V.

TABLE IV

Showing average Calcium balance during 10 days.

Diet.	Number of rats used.	Average food intake. g.	Ca. intake mg.	Ca. in fæces mg.	Ca. % absorbed.	Ca. in urine mg.	Ca. retained. mg.	Ca. % utilised.
Fat-free diet	10	80.2	442.6	97.5	78.9	9.2	335.94	75.8
Butter fat diet	5	74.4	369.8	64.97	82.5	5.96	300.81	80.97
Hydrogenated fat diet	5	70.6	361.1	86.9	76.2	6.01	268.2	74.4

TABLE V

Showing average balance of Phosphorous during 10 days.

Diet.	Number of rats used.	P. intake. mg.	P. in fæces. mg.	P. % absorbed.	P. in urine. mg.	P. retained.	P. % utilised.
Fat free diet	10	513.2	61.83	87.94	86.62	364.74	71.54
Butter fat diet	5	498.1	47.1	90.6	97.7	353.3	70.99
Hydrogenated fat diet	5	480.51	53.18	88.97	100.93	326.4	67.86

It will be seen from the results shown above that when butter fat is added to a fat free ration, there is a favourable influence on calcium absorption. The addition of hydrogenated vegetable fat, on the other hand, shows no beneficial effect on calcium absorption. Hydrogenated vegetable fat induces a greater loss

of calcium, in feces as compared to butter fat. The addition of fat to a fat-free ration, in general tends to lower the urinary excretion of calcium. This effect was observed with the use of both butter fat and hydrogenated fat in the diet. The results of phosphorous balance shown in table V indicates that there is no significant difference in the absorption of phosphorous in the two groups. The urinary excretion of phosphorous is slightly increased on diets containing fat.

Comparison of the effect of butter fat and hydrogenated vegetable fat on the absorption of calcium and phosphorous in diets deficient in vitamins A and D (without the use of Cod-liver oil).

The experimental method, was in general, the same as that described in the first experiment. The composition of the diets was also the same, the only difference being the omission of the supplements of A and D. Six young healthy male albino rats were used for the test. All the rats were initially fed on the fat-free diet supplemented with 60 mg. of unsaponifiable matter of cod-liver oil per rat per day. The purpose of administering unsaponifiable matter as a rich source of vitamin A and D along with the fat-free diet in place of customary 2 drops of cod-liver oil in this experiment was to ensure a sufficient supply of vitamin A in rats so that while comparing the effect of two different fats on calcium and phosphorous absorption, the rats may not actually lose in weight. In order that the results may have a quantitative significance, it is necessary that the rats should be in growing condition, as otherwise, the differences in effect on calcium and phosphorous balance, may in reality be due to loss of weight resulting from low consumption of food. Under the condition of the experiment, the rats which received butter fat diet following the fat free diet were in a growing condition during the period when the balance of calcium and phosphorous were studied, even though no supplements of vitamin A and D were given, whereas in the other group of rats which were fed on hydrogenated fat diet following fat-free diet, one animal lost weight while the other two rats were in growing condition. The analysis of total fat, soap, neutral fat and free fatty acids in feces were carried out, the methods used being those of Fowweather's (9) modification of Saxon (10).

RESULTS

Food consumption and gain in weight of the rats is shown in table VI.

TABLE VI

Average food consumption, gain in weight and weight of feces.

Diet.	No. of rats used.	Initial body wt. g.	Final body wt. g.	Average consumption of food per rat in 10 days. g.	Average daily gain in wt. g.	Average wt. of dried feces in 10 days. g.
Fat free diet	6	63.6	93.6	81.03	3.0	1.898
Butter fat diet	3	97.3	132.6	89.54	3.53	2.411
Hydrogenated fat diet	2	104	132	82.9	2.8	3.228
	1	79	74	49.54	— 5	1.885

The differences in the weight of dried faeces on three different diets is significant. On hydrogenated fat diet, the weight of dried faeces was more than that on either fat-free or butter fat diets. This finding is similar to that observed in the first experiment where the diets were supplied with vitamin A and D in the form of cod-liver oil.

The results of calcium and phosphorous metabolic studies are shown in table VII and VIII respectively.

TABLE VII

Showing average calcium balance during 10 days.

Diet.	No. of rats used.	Ca. intake. mg.	Ca. in fæces. mg.	Ca. % absorbed.	Ca. in urine. mg.	Ca. retained. mg.	Ca. % utilised.
Fat free diet	6	447.29	52.33	88.13	8.73	386.02	86.2
Butter fat diet	3	445.23	78.92	82.45	5.93	360.38	81.4
Hydrogenated fat diet	2	448.29	148	66.46	7.94	292.35	64.67
	1	252.65	66.42	73.70	5 07	180.16	71.3

TABLE VIII

Showing the average balance of Phosphorous in 10 days.

Diet.	No. of rats used.	P. intake. mg.	P. in fæces. mg.	P. % absorbed.	P. in urine. mg.	P. retained. mg.	P. % utilised.
Fat free diet	6	514.54	55.83	89.14	80.73	377.97	73.47
Butter fat diet	3	596.61	57.21	90.30	92.23	447.16	74.25
Hydrogenated fat diet	2	597.72	78.92	86.52	112.09	406.7	67.91
	1	336.87	38.82	88.47	62.22	235.83	69.90

It will be seen that there is a considerable difference between butter fat and hydrogenated vegetable fat in their effect on calcium absorption. At practically the same level of calcium intake, the consumption of hydrogenated fat results in a loss of calcium in feces which is practically double that observed in the butter group. A slight lowering of the urinary excretion of calcium as a result of adding fat to a fat-free ration is also observed in the present experiment. Also there has been somewhat better absorption of calcium on fat free ration than on butter fat diet. This is undoubtedly the effect of the administration of unsaponifiable matter of cod-liver oil along with the fat-free diet. Regarding the absorption and retention of phosphorous, there is a slight lowering on hydrogenated fat diet as compared to that obtained on butter fat diet. The difference, however is not so markedly significant as that noticed in the case of calcium. The addition of fat to a fat-free diet results in a slight increase in the urinary excretion of phosphorous.

Table IX shows the amount of soap, fatty acids and fat in the feces.

TABLE IX

Diet.	No. of rats used.	Fat ingested per rat in 10 days. g.	Total fat in fæces. g.	Total soap in fæces as fatty acids. mg.	Neutral fat in fæces. mg.	Free fatty acids. mg.
Fat free diet	3	—	0.325	65.5	168.5	90.0
Butter fat diet	3	13.35	0.729	288.0	201.0	240
Hydrogenated fat diet	3	11.48	0.923	462.0	185.0	276

It will be seen from the above table that the loss of total soap in feces is much greater in the hydrogenated vegetable fat group, than in the butter fat group. This shows that the fatty acids of hydrogenated fat combine with calcium to form more insoluble calcium soaps which cannot be utilised and are thus excreted in the feces. The increased loss of calcium in feces in this group can, therefore, be accounted for in part due to a greater loss of soaps in feces. The loss of free fatty acids and neutral fat in feces, on an average, was prctically the same when the intake of each fat was about 12 to 13 gms. per rat in 10 days, thus showing that both the fats are equally well digested in the rats intestine.

SUMMARY

1. The relative effect of butter fat and hydrogenated vegetable fat at 15 per cent level in the basal diets, on the absorption and retention of calcium and phosphorous in young rats has been studied.

2. The addition of butter fat to a fat-free ration had a beneficial influence on calcium assimilation and it is superior to hydrogented fat in this respect.

3. In the absence of vitamin A and D in the diet, the consumption of hydrogenated fat actually interferes with calcium absorption. Under these conditions there is a loss of calcium in faeces much greater in amounts on hydrogenated fat diet that on butter fat diet. Similarly the utilization of phosphorous is also slightly decreased.

4. The loss of neutral fat and free fatty acids in feces is no less on hydrogenated fat than on butter fat but the loss of soaps in feces is much higher on hydrogenated fat diet than on butter fat diet.

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My thanks are due to Major-General Sir Sahib Singh Sokhey, Kt., I.M.S., Director, Haffkine Institute, Bombay, for his keen interest in this investigation and for his valuable suggestions, and also to the Lady Tata Memorial Trust for the award of a scholarship.

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STUDIES ON THE NUTRITIVE VALUE OF SOYA MILK.
PART I. NUTRITIVE VALUE OF THE PROTEIN.

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(Received for publication, April 6, 1916)

Much work has been done on the nutritive value of soya-bean especially its protein. That the raw protein of the soya-bean cannot support normal growth has been shown by Osborne and Mendel (1) by feeding rats with raw soya-bean protein as the sole source of protein in an otherwise adequate diet. Results of a similar nature have been obtained by determining the biological value and the digestibility coefficient of the protein by the nitrogen metabolism method by several workers. The figures for the biological value obtained by various workers range from 48 to 60 showing that the protein is of poor quality compared to high class animal protein. Aykroyd and Krishnan (2) showed that it had no beneficial supplementing effect to a poor rice diet.

The importance of soya-bean protein as an article of food has increased ever since it was shown that the biological value of the protein is very significantly increased by subjecting it to suitable processing—particularly appropriate heat treatment. Since the work of Steenbock *et al* (3, 4) and Wilgus *et al* (5) on the effect of heat treatment on the protein nutritional value of soya-bean, several processed soya products are playing a large part in the dietary of the U. S. A. Several publications have appeared on the protein quality of such processed soya products (Mitchell *et al* 6; Cahill *et al* 7). It is shown that the protein quality of such products both by themselves as well as by their supplementing effect on the more common varieties of food proteins is very high (8, 9).

In the present paper, the nutritive value of soya milk—a processed soya product—has been determined. Recently, Cahill *et al* (7) have studied the biological value of soya milk protein as prepared by Messrs. Borden and Company, on nine human subjects and found a value of 95 compared with egg protein as the standard. The method of preparation of the milk is however not given. In this communication, the nutritive value of the protein of soya-bean milk as prepared in a palatable form (De and Subrahmanyan, 10) in this laboratory has been studied both by the nitrogen balance method and by the rat growth method. The protein value of soya milk has been compared with that of cow's milk as the standard.

Nitrogen Balance Method.

The method and technique used in this experiment were the same as those developed by Chick *et al* (11). Three pairs of healthy adult rats 150—200 grams in weight (3 male and 3 female) were kept in separate metabolism cages and were allowed to get accustomed to the altered surroundings. They were given the ordinary laboratory stock diet during this period. After one week, they were fed *ad libitum* the following basal diet 1. The experimental period lasted for seven days after which the rats were given rest for four days on the laboratory stock diet. Then the second experimental period of seven days began during which period diet 2 was given. After an intermittent period of four days' rest the rats were given the experimental diets 3 and 4 each for one week as usual. The composition of the diets and their preparation are as follows:—

Diet 1:—Cocoanut oil 10%, Osborne and Mendel salt mixture 4%,* Vitaminised starch 5%, sugar 10% and starch 71%.

Diet 2:—Cow's milk was concentrated in aluminium pans to $\frac{2}{3}$ rd. the original volume and incorporated with a mixture of starch, cane-sugar and salts such that the composition of the mixture became on a dry basis 10% protein, 4% salts, 10% sugar, 71% starch and fat and 5% vitaminised starch (incorporated later). The milk was analysed for protein and total solids. The whole mass was evaporated

*The vitaminised starch was prepared so as to contain per 100 g., 8 mg. riboflavin, 5 mg. vitamin B_1 hydrochloride, 6 mg. pyridoxine, 2 mg. calcium pantothenate, and 3 mg. choline chloride. The necessary amounts of the vitamins were dissolved in slightly acidulated water, the starch was made into a paste with this solution, and the whole thing was dried in a current of air the temperature not exceeding 60°.

in porcelain basins and heated on the water bath until the moisture content was reduced to about 5%. Then 5% of vitaminised starch on the dry weight of the diet was incorporated. No further fat was added as that in the milk itself was sufficient for meeting the requirements.

Diet 3:—It was prepared in a similar manner to diet No. 2; but here for every 100 cc. of soya milk used 2 g. of cocoanut oil were added.

Diet 4:—It was a mixture of starch, raw soya-bean flour (de-husked), cocoanut oil and salts having the composition: protein 10%, fat 10%, salt 4%, vitaminised starch 5% and the rest carbohydrates.

During each experimental period the rats were given, in addition to the above diets.

1) 70 units of vitamin A (twice weekly).

2) 20 units of calciferol (once weekly).

In the latter half of each experimental period (the last four days) the urine and faeces were collected. The urine samples were preserved with 20 cc. of 5% sulphuric acid containing 2% phenol and 1% thymol. The faeces were collected daily and preserved in the refrigerator. After collection of faeces the metabolism cages and the funnels were rinsed with water into the collecting flasks. The urine samples were filtered, made up to volume and aliquot portions taken for nitrogen estimation. The faecal matter was mixed with 10 cc. of 5% oxalic acid, dried at 100°, powdered well, again dried and weighed. Aliquot portions were taken for the estimation of nitrogen. All nitrogen estimations were made by the semi-micro Kjeldahl method.

From the results obtained, the biological values and the digestibility coefficients for cow's milk protein, soya milk protein and raw soya-bean protein have been calculated according to the usual Chick's formula (11). The results are tabulated below:—

TABLE I

Nitrogen-free diet (0.0081% N)
Figures represent daily averages

Rat No. and sex	Food consumed in g.	Nitrogen intake in mg.	Urinary nitrogen in mg.	Faecal nitrogen in mg.	Biological value	Digestibility coefficient
1M.	7.4	—	34.3	7.9	—	—
2M.	7.1	—	31.9	6.6	—	—
3M.	7.6	—	31.8	7.6	—	—
4F.	8.3	—	43.9	9.8	—	—
5F.	8.5	—	27.5	6.8	—	—
6F.	8.3	—	36.6	6.5	—	—

TABLE II

Cow's milk protein diet
(12-14 mg. nitrogen per gram of food)

Rat No. and sex	Food consumed in g.	Nitrogen intake in mg.	Urinary nitrogen in mg.	Faecal nitrogen in mg.	Biological value	Digestibility coefficient
1M.	14.6	176.6	62.5	40.2	81.5	86.1
2M.	14.2	172.6	60.6	27.3	81.9	92.9
3M.	15.1	183.0	57.5	39.5	85.7	86.6
4F.	11.3	136.8	61.5	29.6	84.7	88.3
5F.	13.3	161.9	55.0	32.4	80.4	86.6
6F.	13.2	160.7	61.3	28.5	82.6	88.7

TABLE III

Soya milk protein diet
(15.58 mg. nitrogen per gram of food)

Rat No. and sex	Food consumed in g.	Nitrogen intake in mg.	Urinary nitrogen in mg.	Faecal nitrogen in mg.	Biological value	Digestibility coefficient
1M.	8.2	127.8	54.7	22.1	82.2	89.7
2M.	9.6	148.3	61.5	23.3	76.3	90.3
3M.	10.5	163.4	68.8	23.1	77.2	91.5
4F.	6.8	106.4	65.0	16.5	78.4	92.0
5F.	9.9	155.4	53.8	10.9	81.6	92.4
6F.	9.9	155.4	65.5	22.4	79.3	89.9

TABLE IV

Raw soya protein diet

Rat No. and sex	Food consumed in g.	Nitrogen intake in mg.	Urinary nitrogen in mg.	Faecal nitrogen in mg.	Biological value	Digestibility coefficient
1M.	13.5	202.5	109.4	47.6	55.6	83.6
2M.	11.3	168.3	97.5	39.9	53.2	82.6
3M.	11.9	179.2	105.1	49.9	53.6	83.3
4F.	9.3	138.4	91.3	25.5	58.1	81.5
5F.	12.8	191.1	97.8	42.7	55.0	83.1
6F.	12.6	188.5	106.3	42.2	55.1	82.9

TABLE V

Comparison of nutritive values

	Cow's milk	Soya milk	Raw soyabean
Biological value	82.8	79.2	55.1
Digestibility coefficient	89.7	90.9	82.8
Nutritive index	74.3	72.0	45.6

Rat Growth Method

Young rats about 50 g. in weight were placed in separate cages and kept for a week on the laboratory stock diet. They were then divided into two groups such that every rat in one group compares with another in the second group with respect to age, litter, weight etc. The rats were then allotted by the toss of coin to soya-bean milk or cow's milk protein diets prepared as follows:—

Freshly prepared soya milk and cow's milk from the dairy were analysed for their nitrogen and total solid contents. Known volume of each milk was evaporated in aluminium pans to ⅔rd the original volume and then transferred to wide porcelain dishes. Cane sugar, starch and salt mixture were then added so that, on the dry basis the mixture contained 10% protein, 4% salts, 81% sugar, starch and fat. In the case of cow's milk no additional fat was added. In case of soya milk for every 100 cc. of soya milk used 2 g. of cocoanut oil were added. The mixtures thus obtained were heated on the water-bath so as to reduce the moisture content to about 5%. 5% vitaminised starch prepared as stated above was incorporated into the diet to supply the source of the water soluble vitamins. The diet thus obtained was stored in the cold room. Fresh food was prepared twice a week. After preparation of the diet, the protein content was checked by drying a representative portion and determining its nitrogen content. The figures for converting nitrogen to protein values were cow's milk 6.25, soya milk 5.7, soya flour 5.7, and taken from Jones (12).

The diet was served daily to the animals and the food consumption was determined daily. The moisture content of the food was also determined every day. The rats were given as usual sufficient amounts of vitamin A and calciferol. The experiment lasted for four weeks. The method of preparation of milk has been described at the end of the paper. The results of the experiment are tabulated below.

TABLE VI

Cow's milk protein

Rat No. and sex	Initial weight in g.	Total food consumed in g.	Total protein consumed in g.	Increase in weight in g.	Increase in weight per g. of protein consumed.
1M.	51	286	26.08	52	1.99
2M.	49	291	27.58	53	1.92
3M.	48	307	27.19	52	1.91
4F.	49	302	26.81	50	1.87
5F.	52	281	26.03	53	2.04
6F.	47	297	26.08	51	1.96

Soya milk protein

Rat No. and Sex	Initial weight in g.	Total food consumed in g.	Total protein consumed in g.	Increase in weight in g.	Increase in weight per g. of protein consumed.
1M.	50	206	19.13	35	1.83
2M.	49	225	19.32	32	1.67
3M.	48	217	18.69	36	1.92
4F.	47	243	19.70	34	1.73
5F.	50	256	20.53	40	1.95
6F.	49	232	19.21	33	1.71

After completion of the above set of experiments it was thought that in the technique of feeding the milk protein some discrepancies may have come in. The heat treatment involved in preparing the diet as described above may have affected the nutritive value of the proteins of the milk. So it was thought necessary to confirm the above results by feeding the milk apart from the basal diet to the rats. The following are the results of the experiments in which the milk was fed seaprately from the basal diet and the protein value determined by observing the growth response of young rats per gram of protein consumed.

Experimental

12 young rats from three litters were chosen and as usual divided into two comparable groups of six in each. The rats in the first group were given cow's milk as the sole source of protein while soya milk was administered to rats in the second group. The amount of milk given was such as to bring the level of protein intake to 8-9%. The basal nitrogen-free diet had the following composition.

Starch	71%
Sugar	10%
Cocoanut oil	10%
Salts	4%
Vitaminised starch	5%

Cow's milk was got daily from the dairy and the soya milk was also prepared every day. The milks were analysed for their nitrogen content and for total solids. Estimations of nitrogen were made once a week on composite samples of milk. The amount of milk administered each day in a week depended on the total consumption of the animals in each week and the milk proteins being fed at a 10% level. As they grew older, their food consumption increased as also the amount of milk administered. Milk and the solid diet were given in separate cups. All the rats consumed the milk measured out to them quantitatively. The total intake of nitrogen in each week was determined. The experiment lasted for six weeks. The total protein intake for the six weeks was calculated from their total nitrogen intake. This gave the total milk proteins consumed by each rat. The rats were weighed every week. Their growth response is presented in Table VII.

TABLE VII

Rat No. and sex	Initial weight in g.	Basal food consumed in g.	Milk solids consumed in g.	Total food intake in g.	Milk protein intake in g.	Total increase in weight in g.	Increase in weight per g. of protein intake.
GROUP I							
1M.	45	208	76	284	21.95	41	1.87
2M.	46	213	,,	289	,,	48	2.19
3M.	47	220	,,	296	,,	45	2.05
4F.	45	216	,,	292	,,	46	2.09
5F.	49	201	,,	277	,,	41	1.87
6F.	42	209	,,	285	,,	41	1.87
GROUP II							
1M.	45	199	51	250	22.59	40	1.77
2M.	44	206	,,	257	,,	41	1.82
3M.	43	226	,,	277	,,	39	1.75
4F.	46	220	,,	271	,,	41	1.82
5F.	42	216	,,	267	,,	42	1.86
6F.	43	210	,,	270	,,	41	1.82

DISCUSSION

A comparison of the biological values of the proteins of cow's milk, soya milk and raw soya-bean would show that the proteins of cow's milk and soya milk have about the same nutritive value. The results also show the marked increase in the nutritive quality of the protein of raw soya bean as the result of processing to yield the milk. As has been observed by other workers the percentage increase in biological value is much greater than the increase in the digestibility coefficient after processing.

The results of table VI give the growth response in the case of the growing rats per gram of protein consumed. The average value in the case of cow's milk and soya milk are 1.95 and 1.80 g. respectively. The nutritive value of the two milks are therefore quite comparable. The results obtained by the growth method are of the same relative order as that got by the nitrogen metabolism method.

In the third set of experiments where the milk is fed separately from the basal diet, results of a similar type are obtained. The average increase in weight per gram of milk protein consumed is 1.99 in the case of cow's milk and 1.80 in case of soya milk. There is not any large difference therefore between the results of the experiments in which the milk was fed mixed with the basal diet and those in which the milk was fed separately.

The results of all the above experiments suggest that soya milk is 90-95% as good as cow's milk regarding its protein value. The usefulness of processing of soya bean in increasing its nutritive value is quite significant.

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Appendix

Preparation of soya-bean milk: Several methods have been described for the preparation of soya-bean milk. The method followed by us for the preparation of milk used in the present research may be described as follows: The soya-beans were soaked overnight in water. The husk was then pealed by gentle rubbing ; the beans were then extracted with 0.04%, sodium bicarbonate at 70° for half an hour. The beans were then made into a fine paste, stirred up with about 6 volumes of water and boiled for 20-30 mins. The mixture is allowed to settle and then filtered through cloth. One pound of soya bean gave 5-6 pounds of milk.

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STUDIES ON THE NUTRITIVE VALUE OF SOYA MILK PART II. COMPARISON OF THE VITAMIN B-COMPLEX CONTENT OF SOYA MILK AND COW'S MILK.

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(Received for publication, April 6, 1946)

In Part I of this communication the protein value of soya milk as compared to that of cow's milk has been determined by the nitrogen balance and the growth methods on rats. In this section the total vitamin B complex content of soya milk has been compared with that of cow's milk. The vitamin B complex content of soya milk as calculated from the amount of these vitamins present in the soya-bean used would not be absolute since some destruction would occur during the boiling of the milk. In this experiment the total B complex vitamins present in the milks

has been determined by growth experiments on rats. The method used is similar to that used by Kon (1) and by Westerman (2). The rats were fed a diet which is otherwise nutritionally adequate and complete except for the B complex vitamins. The soya milk or cow's milk given along with the above diet served as the sole source of the B complex vitamins. The growth response of the rats gave an idea of the vitamin content of the milks.

The soya milk used in this experiment was prepared from soya beans which are allowed to germinate for 24 hours. This was done because germination is well known to increase or release the content of some of the B complex vitamins in a number of pulses. Recently French *et al* (3) have determined the effect of germination on the B complex vitamin content of soya beans, pea and other beans. Their results are quite interesting in that there is a marked increase in the B complex vitamins during germination. Moreover, the protein of germinated soya beans is shown to have a higher biological value than the raw beans by Everson, Steenbock *et al* (4). The milk obtained from the germinated product was found to have a better flavour and taste than the milk from the ungerminated sample.

The cow's milk used was got daily from the dairy. The milk was just boiled for 5 minutes, cooled and served to the animals. During this process exposure to the air was minimised as much as possible. Boiled milk was used for comparison rather than raw unheated milk because milk is generally used in India only after boiling.

Experimental

18 rats, 40-50 g. in weight, were taken and were divided into three groups of six in each group so that the rats in the three groups compared with one another with respect to weight, litter, sex, etc. Group I served as the control over groups II and III which got as their sole source of the B complex vitamin cow's milk and soya milk respectively. All the rats were given the following diet as well as adequate supplements of vitamin A and calciferol.

The composition of the basal diet is as follows:

Starch	57%
Sugar	10%
Salts	4%
Groundnut oil	5%
Casein	24%

The casein used in the experiment was prepared from ordinary commercial casein which was washed several times with water adjusted to *p*H 4.6 (iso-electric point of casein) to wash away all the B complex vitamins in it.

All the rats were given the basal diet only for two weeks. For the first ten days all the rats increased in weight but in the latter half of the second week all the rats showed a definite decrease in weight showing that they had been run out of their body store of the B complex vitamins. The rats were then weighed and rats in groups II and III were given henceforth in addition to the above diet 8 cc. of cow's milk and 10 cc. of soya milk respectively. To the rats in group I the basal diet was continued. The rats were weighed weekly and fresh food was given daily. The experiment lasted for six weeks after the period of milk administration began The growth response of the rats has been tabulated below.

The rats in the group I showed a continued decrease in weight and died on account of vitamin B complex deficiency within a period of 30-40 days from the beginning of the experiment.

Rat No. and sex	Initial weight in g.	Weight when milk supplementation began in g.	Final weight in g.	Increase in weight in g.	Difference in weight between pairs.
1M.	43	57	97	40	
1M.	51	60	104	44	− 4
2M.	43	50	104	54	
2M.	41	51	99	48	+ 6
3M.	40	45	108	63	
3M.	40	46	96	50	+13
4F.	40	48	85	47	
4F.	39	45	97	52	− 5
5F.	41	46	84	38	
5F.	41	50	94	44	− 6
6F.	50	59	97	38	
6F.	51	59	95	36	+ 2
					Total difference + 6

From the above figures tabulated above it can be seen that there is no significant difference in the growth response of the rats due to administration of soya and cow's milks as the source of the B complex vitamins. From the results it can be said that soya milk is about 80% as potent as cow's milk with regard to its vitamin B complex content. It is to be noted that the results of this experiment do not give an idea about the content of the individual vitamins in the milks. It gives an approximate idea of the general efficiency of the milks as sources of the vitamin B complex.

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STUDIES ON THE NUTRITIVE VALUE OF SOYA-BEAN MILK. PART III. SUPPLEMENTING VALUE OF SOYA MILK TO A POOR INDIAN DIET

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The relative nutritive value of typical Indian diets has been assessed by growth experiments on experimental animals. While investigating the nutritive value of a South Indian diet, Aykroyd and Krishnan (1) showed that a South Indian diet is very poor nutritionally. They tried supplementing the poor South Indian diet with a variety of food products (2, 3). They used as supplements skimmed milk

powder, eggs, fish, arhar dal, black-gram, soya-bean etc. They showed that soya-bean either cooked or raw produced no marked gain in weight over the control set of rats receiving no supplement. Soya bean did not produce a beneficial effect perhaps owing to its poor quality protein in the form in which it was used in their experiments. But the importance of processing of soya bean in raising the nutritive value of the protein is however coming to be recognised increasingly. Recent publications about the protein nutritive value of processed soya products, show that suitable processing can increase the protein value to a degree quite comparable to that of any high class animal protein (4). The supplementing value of processed soya flour to the proteins of other commoner foods has also been determined. Jones and Divine showed that addition of even as small a quantity as 5% of soya flour to wheat flour, increased very much the growth promoting value of the mixed proteins of the flour (5). More recently Francis *et al* (6) have reached a similar conclusion while investigating the effect of soya flour on the nutritive value of the protein of white bread.

In Part I of this communication, while comparing the nutritive value of soya milk protein with that of cow's milk, it has been shown that the protein of soya milk has quite a high biological value comparing well with that of cow's milk. It was therefore considered important to study the supplementng value of soya milk to a poor South Indian diet. The results of such a study performed on rats are described in this section.

The poor South Indian diet chosen in this experiment is essentially the same as that chosen by Aykroyd and Krishnan (1). It had the following composition:—

Raw Rice	21.0
Dal	0.7
Blackgram	0.7
Gingelly oil	0.1
Cocoanut oil	0.05

Experimental

A group of 18 rats was chosen from among 4 litters and they were divided into three groups of six in each so that the rats in the three groups were comparable with respect to weight, litter and sex. Group I served as the control set receiving the basal diet described above. Groups II and III received along with the basal diet supplements of soya milk. Group II received 7 cc. of soya milk and rats in the third group received 10 c c. of soya milk daily as supplement. The soya milk was prepared daily and served fresh to the animals. The basal diet was fed *ad libitum* to all the rats in the three groups. In addition to the above all the rats received the following vegetables (3 g. of Brinjals and 3 g. Amaranth leaves to each rat twice weekly).

The growth response of the rats was followed. The rats were weighed weekly and their food consumption was determined. The experiment lasted for seven weeks. The average weekly increase in weight has been calculated. The results of the experiment are tabulated below:

Rat No. and sex	Initial Weight in g.	Food consumed in g.	Increase in weight in 7 weeks in g.	Average weekly increase in weight.
GROUP I				
1M.	44	217	17	
2M.	46	212	25	
3M.	48	227	22	2.8 g.
4F.	43	217	17	
5F.	48	204	19	
6F.	42	222	17	
GROUP II				
1M.	40	234	30	
2M.	54	238	32	
3M.	48	230	29	4.1 g.
4F.	41	225	28	
5F.	39	230	32	
6F.	43	221	21	
GROUP III				
1M.	48	251	33	
2M.	46	262	53	
3M.	45	256	44	5.4 g.
4F.	48	247	34	
5F.	43	242	34	
6F.	40	234	31	

It will be clear from the results tabulated above that a small amount of soya-bean milk given as a supplement increases the average weekly increase in weight from 2.8 g. to 5.5 g. In the case of the experiments of Aykroyd and Krishnan the rate of growth on a soya bean supplement is 4.6 g. week either with cooked or raw beans. The above results show that soya milk is superior to raw soya-bean as a supplement to the poor rice diet. This is perhaps due to the higher biological value and digestibility co-efficient of the protein of soya milk than those of raw soya bean.

It is to be noted that the rate of growth of the rats even when supplemented with soya-bean milk is by no means optimal. This is due to the fact that the diet even after supplementing with soya milk is not adequate and complete. It is however shown that soya-bean supplemented to a poor rice diet which is further supplemented with yeast extract and calcium lactate can promote the normal growth of rats (7, 8). The experiments described in this section show however that soya milk supplemented to a poor rice diet can enhance the rate of growth of the rats by a significant amount.

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STUDIES ON THE NUTRITIVE VALUE OF SOYA MILK. PART IV. DIGESTIBILITY OF SOYA MILK—'IN VITRO' EXPERIMENTS

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Soya-bean has been known as an article of food in China and other Far-Eastern countries from a long time. It has also been used as an infant food (1).

Recently, it has been shown, by experiments on rats, using the balance sheet method (2, 3) that the soya-bean can be best utilised in the form of milk. There are three methods available by which the digestibility of milks can be determined and they are (*a*) *in vivo* experiments ; (*b*) *in vitro* experiments and (*c*) curd tension measurements. It has been shown from *in vivo* experiments, that soya milk is as digestible as cow's milk. Since the *in vivo* digestibility is influenced by a number of unknown factors and since the experiments are time-consuming, it was considered desirable to determine the digestibility of the milk by the *in vitro* method—a method which aims at repeating the processes occurring in the gastro-intestinal tract, the main limitation being the lack of a wide variety of enzymes, coenzymes and accelerators which are brought into play by the latter. If the *in vitro* digestibility could bear any proportionality to the *in vivo* findings, it could then be used with greater facility for routine testing. It could also be used for following up the efficacy of the different steps that are used in the processing of the bean.

Experimental procedure:—The procedure for following the rate of digestibility was similar to that employed by Turner for milks of different kinds. Briefly the method, as followed by us, may be outlined as follows.

For the experiments soya-milk was prepared according to the method described in an earlier paper (2). 400 cc. of the sample of milk were taken in a conical flask and maintained at 37°. In another flask 40 cc. of N/2 hydrochloride acid were placed and to it 2 cc. of cow'e stomach mucosa extract were added (standard pepsin was not available and as a source of pepsin a 0.05N hydrochloric acid extract of fresh cow stomach mucosa was used). The milk was transferred into the acid solution as quickly as possible with thorough shaking. The *p*H of the resulting mixture was always below 3.6.

The flask was maintained at 37° and the time of mixing the milk and the enzyme was noted. Five minutes later, 25 cc. of the digest were measured out and filtered as quickly as possible in order that the amount of soluble protein may not increase with the time of filtration. The same procedure was adopted at the

end of half an hour from the time of mixing. The filtrates were kept aside for protein determination by the macro-Kjeldahl method.

All this while the digestion was taking place in an acid medium, *i.e.*, facilitating peptic digestion. In order to carry out the tryptic digestion on the same sample after peptic digestion, it was necessary to change the medium to the alkaline side. This was effected by using alkali and the *p*H was adjusted to 7.5 by careful addition of alkali. It is well known that using both pepsin and trypsin on the same sample, a larger percentage of protein can be extracted.

On the addition of alkali, the colour of the digest change to yellow. The time of adding the alkali was noted and 5 minutes later, 6 cc. of pancreas extract were added (as a source of trypsin, a 20% glycerol extract of fresh beef pancreas was used). The solution was stirred well. 15 minutes later, 25 cc. of the digestion mixture were taken and about 2-3 cc. of N-HCl were added till the *p*H came down to 4.7 ± 0.1. On adding the necessary volume of acid, the insoluble protein was precipitated and the acid stopped further digestion by the enzyme. The solution was filtered and the protein estimated in the filtrate. Similarly filtrations were carried out at intervals for a period of 3 hours.

The total protein in the milk as well as the solid content were determined for each sample of milk analysed. Side by side with these experiments, control experiments using pasteurised cow's milk were also carried out.

Since the enzyme solutions used were not the same throughout, they were standardised daily by the formol titration method using a substrate of standard casein solution. The results are given in Table I.

TABLE I

Digestibility of soya milk prepared by germinating soya beans for 48 hours.

Time in minutes.	% protein in solution.	% acid insoluble protein converted to acid soluble.	Total protein content.	Solid content.
5	0.17	0.0		
20	0.17	0.1		
42	0.54	9.5	4.0%	8.5%
120	—	—		
180	0.61	12.1		
	Digestibility of raw soya powder.			
5	0.55	0.0	3.92%	
180	0.74	5.7		
	Digestibility of pasteurised cow's milk.			
5	0.55	0.0		
30	0.61	2.3		
70	1.00	16.6	3.25%	
115	1.71	42.7		
180	2.14	58.4		

Experiments on the inhibitor in soya-beans:—Recently it has been suggested that soya-bean contains a proteolytic inhibitor which is probably a polypeptide (5). It is considered that this inhibitor is identical with a factor in soya-beans which retards the growth in chicks. Preliminary work was carried out in this laboratory to verify the presence of the inhibitor.

According to Ham and Sandstedt (5) this inhibitor is extractable from unheated soya meal at *p*H 4.2 at which reaction the bulk of the soya proteins is insoluble. Further they have shown that when 5 cc. of a dilute acid extract of the unheated meal are added to trypsin on a casein substrate, the action of trypsin is retarded as shown by formol titration figures.

In a similar manner, soya-bean was soaked overnight in a dilute acid solution of *p*H 4.2 and 5 cc. of the extract were added to the substrate of 20 cc. 5% casein solution containing 2 cc. of the trypsin extract. The *p*H was adjusted to 8.0 and the solution was incubated at 40° after adding toluene as a preservative. The results show that compared to a standard substrate containing casein and the enzyme alone at *p*H 8.0, the value got in the presence of the acid extract was distinctly lower. This confirms the presence of the inhibitor postulated by Ham and Sandstedt (*loc. cit*).

TABLE II.

Experiments on the inhibitor.

(*a*) Trypsin alone.

20 cc. 5% casein +2 cc. trypsin extract (*p*H 8)

Time in hours.	Vol. of N/50 alkali (formol titration)	Amount of nitrogen in solution in 3 hours.
0	0.9 cc.	
2	3.4 cc.	0.84 mg.
3	3.9 cc.	

(*b*) Trypsin + Inhibitor.

20 cc. 5% casein + 2 cc. trypsin + 5 cc. trypsin inhibitor solution (*p*H 8)

Time in hours.	Vol. of N/50 alkali (formol titration)	Amount of nitrogen in solution in 3 hours.
0	1.15 cc.	
1	2.1 cc	
2	2.6 cc.	0.434 mg.
3	2.7 rr.	

Method of calculation:—The difference between the total protein content of the milk and the amount of milk protein soluble at *p*H 4.7 (5 minutes reading) was taken as the acid insoluble protein. The percentage of protein in 5 minutes was substracted from the other values at the end of ½ hour etc., till 3 hours and this

divided by the total acid insoluble protein gives the percentage of acid insoluble protein converted to the acid soluble condition. The rate of digestion of the acid insoluble protein was used as an index of milk digestibility.

Results and discussions:—A study of the rate of digestibility of soya milk compared to cow's milk shows that the former is poorly digested. This is contrary to the findings of the *in vivo* experiments (2). As already stated, the presence of a proteolytic inhibitor system in the soya-bean has been confirmed and whether or not it is solely responsible for the low digestibility of soya milk *in vitro* is a question yet to be solved. Further work on similar lines using activators etc., and methods using curd tension measurements are under way.

SUMMARY

1. Although the *in vivo* digestibility of soya bean milk is of the same order (about 90%) as that of cow's milk, its *in vitro* digestibility as measured by peptic followed by tryptic hydrolysis is only about one-fifth of that of the latter.

2. The raw bean contains a weak trypsin inhibitor, but its action does not fully explain the low *in vitro* digestiibility.

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IMPROVEMENTS IN THE CYANOGEN BROMIDE METHOD FOR THE DETERMINATION OF NICOTINIC ACID IN URINE

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A method for the estimation of nicotinic acid in urine using the cyanogen bromide-aniline reagent (Königs reagent) was first described by the author in 1939 (1). Other workers (2-5) have proposed certain modifications in this method. In a recent publication the author (6) adduced evidence to show that in general these modifications do not lead to more satisfactory results than the technique published by the author in 1939 ; however, they have helped to bring out the various sources of error that are likely to occur in the different modifications of the cyanogen bromide method and the precautions necessary for obtaining reliable results. The chief sources of error are (1) allowance of a correct value for the blank *i.e.*, the colour originally present in the extract, (2) the side reactions of the aromatic amine—especially in strong acid and/or alcoholic medium and (3) the side reactions of the cyanogen bromide—especially when the solutions are heated at 70°C with CNBr before the production of colour and (4) the partial conversion of trigonellin to nicotinic acid during hydrolysis with strong alkali in the presence of urea or ammonium salts.

The author has already shown (Swaminathan *loc cit*) that the errors involved in allowing a value for the 'blank' and also due to the side reactions of the aromatic amine and cyanogen bromide are avoided or reduced to the minimum if the colour is developed in the cold in aqueous medium at *p*H 7 without preliminary heating with cyanogen bromide at 70° and that hydrolysis of the urine with N. NaOH does not convert any trigonelline to nicotinic acid.

More recently Wang and Kodicek (7) have made a detailed investigation of the cyanogen bromide method as applied to urine and have introduced certain

modifications for obtaining reliable results. But their method is rather long, involving too many manipulations and hence not suited for routine use in clinical laboratories. In the present paper a simplified procedure for the determination of nicotinic acid in urine is described. The method has been used in this laboratory during the last 3 years and has proved quite satisfactory. It is simpler and less time consuming than the technique described by Wang and Kodicek (*loc. cit*). By following this method, a single worker can determine the nicotinic acid content of 20 to 24 samples of urine in a day.

EXPERIMENTAL

Reagents required

1. *Standard Nicotinic acid (strong)* 1 ml. = 1 mg. nicotinic acid dissolved in N/10 HCl and kept in a refrigerator (a few drops of toluene is added as a preservative). This standard keeps well for nearly a year.

2. *Standard Nicotinic acid* (*dilute*) 1 ml. = 20 micrograms of nicotinic acid prepared fresh as required by diluting 1 ml. of solution (1) to 50 ml. with distilled water.

3. *Aqueous cyanogon bromide solution.* This is prepared fresh by decolourising an ice cold saturated solution of bromine water by the gradual addition of 20% sodium cyanide.

4. Aqueous aniline 2% prepared fresh by dissolving 2 ml. of freshly distilled aniline in 80 ml. of distilled water and diluting to 100 ml.

5. Sodium acetate 50% aqueous solution (adjusted to *p*H 7).

6. Sodium hydroxide 4%.

7. Zinc Chloride 20% sol.

8. Sodium hydroxide (4% approx.) (The strength is so adjusted such that 6 cc. of the solution when added to 2 cc. of 20% Zinc chloride solution will precipitate completely Zinc hydroxide and the supernatant fluid will be very faintly pink to phenolphthalein.)

9. Barium acetate 20% sol.

METHOD

The method consists of the following steps:—

1. Hydrolysis of nicotinic acid derivatives (*i.e.*) nicotinamide and nicotinuric acid, to nicotinic acid by heating at 100°C with N.NaOH for 45 minutes.

2. Decolourisation of the urine with Zinc hydroxide at *p*H 9.5—10 and adjustment of the *p*H of the filtrate to 7.

3. Colorimetric estimation of the nicotinic acid present in aliquots using cyanogen bromide-aniline reagent.

PROCEDURE

1. *Hydrolysis of nicotinic acid derivatives to nicotinic acid.*

Fifty ml. of urine were measured into a 250 ml. beaker. The urine was made just alkaline to phenolphthalein (as internal indicator) by the addition of 40% NaOH. Five ml. of 40% NaOH were then added and the mixture heated on a steam bath for 45 minutes. (12 or 24 samples could be started at the same time).

2. *Decolourisation of the urine with zinc hydroxide.*

The urine after hydrolysis was cooled and neutralised by the addition of concentrated HCl so that the extract has a *p*H of 7 to 7.4. Two ml. of 20% zinc chloride solution and 5 ml. of 20% barium acetate solution were then added. Zinc hydroxide was precipitated by the careful addition of 4% sodium hydroxide with phenolphthalein as internal indicator, so that the mixture was very faintly pink in colour (about 6 ml. of sodium hydroxide may be required and excess of alkali should be avoided). The mixture was then made up to volume (50 or 100 ml. depending on the nicotinic acid content) and filtered. The filtrate was adjusted to *p*H 7 by the addition of a few drops of concentrated and dilute HCl as required. Aliquots were used for colorimetric estimation of the nicotinic acid present. The extracts had a light yellow colour, which was allowed for by a 'blank' estimation.

3. *Colorimetric procedure*

Aliquots of the extract (usually 10 ml.) were measured into a series of 25 ml. measuring flasks. Standard nicotinic acid (20 micrograms) was taken in another flask and the volume of the standard diluted to 10 ml. with distilled water. One ml. of 50% sodium acetate solution (adjusted to *p*H 7.4) was then added to each flask. Two ml. of aqueous aniline solution were then added to all the flasks, followed by 7 ml. of cyanogen bromide solution. The contents of the flask were mixed and allowed to stand for one minute. The volume was then made up to 25 ml. by the addition of aqueous aniline, the solutions well mixed and allowed to stand for one minute. The colours were compared immediately (within 5 minutes) in a Klett colorimeter.

A blank estimation was carried out in the above manner for all the coloured extracts with the difference that distilled water was added instead of cyanogen bromide and aniline solutions. The values so obtained for the 'blanks' were allowed for in the usual manner.

Recovery of nicotinic acid added to urine

Different known quantities of nicotinic acid were added to measured volumes of different samples of urine. The recovery of added nicotinic acid was good in all the cases. The results are given in Table I.

TABLE I

Recovery of nicotinic acid added to urine

Experiment No.	Urines with and without added nicotinic acid.	Total Nicotinic acid found.	Recovery (per cent)
I.	Urine (A) 50 ml.	0.054	...
	Urine (A) 50 ml. +0.05 mg. nicotinic acid	0.110	110
II.	Urine (B) 50 ml.	0.065	...
	Urine (B) 50 ml. +0.10 mg. nicotinic acid	0.160	95
III.	Urine (C) 50 ml.	0.085	...
	Urine (C) 50 ml. +0.10 mg. nicotinic acid	0.180	95
IV.	Urine (D) 50 ml.	0.075	...
	Urine (4) 50 ml. +0.10 mg. nicotinic acid	0.165	90

Effect of different decolorising procedures on the value of the blank and the nicotinic acid values

Fifty ml. samples of urine were hydrolysed according to the procedure given in this paper. Decolorisation was effected by following three different procedures: (1) the old procedure of the author using charcoal ; (2) the present method using zinc hydroxide and (3) according to the procedure of Wang and Kodicek (7) *i.e.* oxidation with potassium permanganate after washing with isobutyl alcohol. The final solutions were adjusted to *p*H 7 and made up to 80 ml. Ten ml. aliquots were used for the estimation of the nicotinic acid present. The results are shown in Table II.

The data given in Table II show (1) charcoal or Zinc hydroxide does not absorb nicotinic acid from urine extracts under the conditions given in this paper, (2) the residual colour present in the extracts does not interfere when allowed for as a blank under the conditions described by the author, (3) Zinc hydroxide decolourises urine extracts more efficiently than charcoal or oxidation with potassium permangnate after preliminary washing with isobutylalcohol as recommended by Wang and Kodicek (*loc. cit*). (4) Similar values are obtained for the nicotinic acid content of urine by following the three different decolourising procedures and (5) when aqueous aniline is employed at *p*H 7 for the production of colour the "amine blank" has the same value as the "dilution blank."

TABLE II

Effect of different decolorising procedures on the value of the blank and the nicotinic acid

Decolorising procedure.	Name of coloured solution	Colorimetric readings (scale divisions) Standard nicotinic acid (20 μg.)	Colorimetric readings (scale divisions) Unknown solution	Strength of unknown in terms of nicotinic acid (microgram)	Nicotinic acid found in 10 ml. of solution (microgram)
Charcoal according to Swaminathan (1939)	Test	40	25.4	31.5	—
	Aniline blank	20	38.6	10.4	21.1
	Dilution blank	20	39.2	10.2	21.3
Zinc hydroxide (present paper)	Test	40	28.5	28.1	—
	Aniline blank	15	44.2	6.8	21.3
	Dilution blank	15	44.6	6.7	21.4
Washing with isobutanol and oxidation with KMNO4 according to Wang and Kodicek 1943	Test	40	27.0	29.6	—
	Aniline blank	15	36.4	8.2	21.4
	Dilution blank	15	37.0	8.1	21.5

The urinary excretion of nicotinic acid in hospital patients

The method was applied to the estimation of nicotinic acid in the urine of a group of hospital patients. This group included seven starving sick destitutes admitted for treatment, one case of anaemia and another of incipient pellagra. The total nicotinic acid excreted was estimated in 24 hour samples of urine before and after the administration of 100 mg. of nicotinic acid. Ten ml. of glacial acetic acid and 5 ml. of toluene were added as preservatives to the bottles used for the collection of urine. If the total amount of urine excreted each day was less than 1 litre, it was diluted to 1 litre and 50 ml. used for the assay. The results are given in the Table III.

TABLE III

Urinary excretion of nicotinic acid in hospital patients before and after administration of nicotinic acid

Number of subject	Diagnosis	Initial excretion in 24 hours (mg)	Excretion after a test dose of 100 mg. in 24 hours (mg.)
1	Starvation	1.5	2.1
2	,,	1.5	6.5
3	,,	0.8	1.2
4	,,	1.8	8.9
5	,,	1.6	3.3
6	,,	1.0	2.7
7	,,	1.2	1.8
8	Anæmia	1.8	15.0
9	Incipient pellagra	2.2	3.6

The results show that the response to a test dose of 100 mg. was very low in the starving sick destitutes, when compared to that obtained before by the author for malnourished hospital patients (1).

SUMMARY

The cyanogen bromide-aniline method for the estimation of nicotinic acid in urine has been further improved. The modified method consists of three steps (1) hydrolysis of nicotinamide and nicotinuric acid to nicotinic acid with N. NaOH by heating on a steam bath for 45 minutes, (2) decolorisation of the hydrolysed urine with zinc hydroxide, (3) colorimetric estimation of nicotinic acid in aqueous medium at *p*H 7 using cyanogen bromide and aqueous aniline. The method is simpler and less time consuming than the techniques described by other workers. As many as 24 samples of urine can be assayed in a day by a single worker.

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NUTRITIONAL INVESTIGATIONS ON BENGAL FISH

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In our previous communications (1-6) we have given the analysis of 60 different varieties of Bengal fresh-water fish for water, body-fat, ash, protein, calcium, phosphorus, total and ionizable iron, copper, nicotinic acid, vitamin A and D. The above investigations were carried out in fresh raw muscle tissues of fish available in Calcutta market.

Now, as in some parts of Bengal, sun-dried and salted fishes are also available in quantities and consumed by the people and owing to the seasonal gluts a very large quantity of fish is allowed to perish every year, the nutritional values of these preserved fishes deserve systematic investigations.

The present communication gives the results concerning the moisture, body-fat, total mineral matter, protein, total iron, calcium and phosphorus content of different varieties of sun-dried fish, obtained from the local market. Further work on this line are in progress and the results will be published in subsequent communications.

EXPERIMENTAL

The methods adopted in these investigations are the same as described in our previous papers (1-6). The mean values given in Tables I and II are based on the analysis of three to four samples of each variety of fish. The zoological names of a few of them have not been obtained.

TABLE I

Mean values of water, body-fat, protein and ash per 100 g. of sun-dried fish.

Bengali name.	Zoological name.	Water. (g.)	Body-fat. (g.)	Protein. (g.)	Ash. (g.)	Ranges of body weight in. (g.)
Vetki	*Lates calcifer*	20.10	1.95	60.15	15.90	52-68
Laukhola	... ...	33.20	5.38	49.76	6.57	28-61
Bombay duck	... ...	17.40	5.48	59.85	12.11	...
Fesha	*Engraulis lelara*	10.30	4.92	70.87	11.91	4-34
Parshey	*Mugil parsia*	11.82	4.38	64.95	15.82	1-8
Bhangar	*Mugil tade*	17.92	2.26	61.48	16.47	4-26
Chingri (Small)	... ...	17.86	3.87	62.36	13.87	0.5-2
Muti	... ...	19.15	2.41	63.61	11.76	1-2
*Hilsa	*Clupea ilisa*	55.9	11.81	14.09	16.48	...

*The fish Hilsa has been preserved by wet curing with salt.

TABLE II

Mean values of Calcium, Phosphorus and Iron in mg. per 100 g. of fish.

Bengali name.	Zoological name.	Calcium.	Phosphorus.	Iron.
Vetki	*Lates Calcifer*	938.82	346.98	14.97
Laukhola	... ...	514.50	292.60	17.75
Bombay duck	... ...	1389.70	240.44	19.14
Fesha	*Engraulis lelara*	1676.15	477.57	18.00
Parsey	*Mugil Parsia*	2231.20	396.40	17.42
Bhangar	*Mugil tade*	623.48	207.20	9.32
Chingri	... ...	3538.50	353.60	27.90
Muti	... ...	724.00	833.80	1.00
Hilsa	*Clupea ilisa*	542.68	437.20	11.60

SUMMARY

Among the fish analysed Hilsa (*Clupea ilisa*) has the highest fat content, 11.81 per cent, then come Bombay duck and Laukhola, 5.48 and 5.38 per cent respectively. The protein content, 70.87 per cent is the highest in Fesha (Engraulis lelara). The value of calcium, 3538.50 mg. per cent, is the highest in Chingri. The next best sources of calcium are Parsey and Fesha which contain 2231.20 and 1676.15 mg. per cent of calcium respectively. The phosphorus content. 833.80 per cent, is highest in Muti. As regards total iron, Chingri is the best source containing 27.98 mg. per cent. Greater percentage of ash content in some of the samples, as compared to our previous findings with fresh raw fish indicates associated impurities other than the nutritive mineral constituents present in the fish. These impurities are probably sand and silica, which are invariably present due to the non-hygienic method of drying the fish on sand-bed near the sea or river. Higher percentage of water than the usual in dehydrated fish (10-15%) decreases the keeping quality of fish. In some cases products are seemed to be unfit for human consumption. So some improvements on the existing method of manufacturing the sun-dried (sutki) fish seems to be urgently needed.

Our best thanks are due to Prof. B. C. Guha and Dr. B. N. Ghosh for their advice and interest and to the Indian Research Fund Association for a research grant.

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VITAMIN A RESERVE IN HUMAN LIVER

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Large amounts of Vitamin A are stored in the liver. Vitamin A is rapidly deposited in the liver when optimal or excessive amounts are given, while no vitamin is stored during low vitamin intake (Lewis *et al,* 1). The stored vitamin is used rapidly when the food intake is deficient until a certain apparently critical vitamin A level in the liver is reached ; thereafter, the rate of depletion is slow (Davies & Moore, 2). In many diseases, such as fever, an increased demand for vitamin A has been demonstrated (Clausen, 3). Moor (4) and Myburgh (5) have shown that vitamin A content of the liver of rats decreased rapidly and finally disappeared completely when the animals were kept on a vitamin-free diet. There are no quantitative data available which would indicate what the minimum amount stored in the human liver should be.

This paper deals with the vitamin A reserve in 16 human livers. It covers 6 cases of sudden death (within a few hours) caused in accident and 8 pathological cases. One case was presumably of narcotic poisoning and one of still birth.

METHOD

The blue colour developed by the unsaponifiable fraction (obtained by the alkali digestion method of Davies (6) with $SbCl_3$ was compared in a Rosenhein tintometer. In most of the cases duplicate readings of the blue colour were taken after a little modification made in the extraction of the unsaponifiable fraction as described below.

5 g. minced liver were digested with 10 ml. of 5 per cent KOH in a small conical flask containing 5 ml. of alcohol till complete solution was obtained. It was rinsed to a separating funnel with 20 ml. of water and 5 ml. of alcohol and shaken carefully twice with 40 and 30 ml. of chloroform respectively. The combined chloroform extract was first washed with 5 ml. of water to remove the soap and then with 40 ml. of slightly acidulated water and finally with 40 ml. of distilled water. The chloroform extract was dried over A. R. anhydrous sodium sulphate for an hour, filtered and the solution distilled to a bulk less than 5 ml. The blue colour with $SbCl_3$ was determined as usual. However it was found that chloroform extracts besides vitamin A other yellow pigments such as bile salts, bilivirdin, etc., which interfere in exact matching of the blue colour. The comparative values by the two methods are not given here because they were identical in most of the cases.

RESULTS

Table I gives the details of the findings. The results, which were originally determined in Carr-Price units, were also converted to other units, though such conversion is far from an accurate expression. The calculated results are given in Table II.

1 Carr-Price (or 1 Lovibond Unit)	= Tintometer blue units given by 0.2 ml. of 20% solution in chloroform in 2 ml. of saturated solution of $SbCl_3$ in chloroform.
Moor's Blue Unit	= The intensity of blue colour in Tintometer units developed by 1 gm. of the substance in 1 ml. volume.
1 Lovibond Unit	= 55 Moor's blue unit.
	= 20.8 I.U.
	= 12.5 γ B-Carotene
	= 0.013 $E^{1\%}_{1\,cm.}$ 328 m μ

TABLE I

Vitamin A reserve in Liver.

Serial No.	Cause of Death.	Age	Weight of fresh liver.	Moor's blue units. per g. of fresh liver.	Moor's blue units. Total
1.	Brain fracture in lorry accident; sudden death	35	1306 g.	687	897,496
2.	—do— —do— —do—	9	568	440	249,920
3.	—do— —do— —do—	30	1985	330	654,060
4.	Railway accident; sudden death	22	1462	715	1,045,340
5.	Accident, death within few hours	30	1402	198	277,596
6.	Death due to injury in an accident	31	1246	550	479,710
7.	Malaria B.T.	—	—	275	—
8.	Tetany	—	992	121	120,032
9.	Amoebic dysentery	—	1132	Traces.	Traces.
10.	Broncho-pneumonia	—	—	82.5	—
11.	,, ,,	—	1963	66	129,558
12.	Lobar-pneumonia	—	1472	12	17,664
13.	,, ,,	—	1586	Traces.	Traces.
14.	Pleural adhesive	—	1485	88	63,340
15.	Still birth	—	170	Traces.	Traces.
16.	Suspected Narcotic poisoning	25	1133	440	498,520

TABLE II

Vitamin A content of the fresh human Liver.

Serial No.	Moor's Blue Units.	Carr-Price Value.	I. U. Per G	β-Carotine γ per G.	$E^{1\%}_{1\ cm.}$ 328 $m\mu$
1.	687	12.5	260	156	0.1625
2.	440	8.0	166	100	0,1040
3.	330	6.0	125	75	0.0781
4.	715	13.0	130	162	0.1690
5.	198	3.6	75	35	0.0168
6.	550	10.0	208	125	0.1300
Mean	485	8.8	161	108	0.1150
7.	275	5.0	104	63	0.0650
8.	121	2.2	46	28	0.0285
9.	Traces	—	—	—	—
10.	82.5	1.5	31	19	0.0195
11.	66	1.2	25	15	0.0156
12.	12	0.22	5	3	0.0029
13.	Traces	—	—	—	—
14.	88	1.6	33	20	0.0208
15.	Traces	—	—	—	—
16.	440	8.0	116	100	0.1040

NOTE:—Serial Nos. are the same as in Table I.

Summary

1. Vitamin A reserve in 16 human liver is described.

2. The average vitamin A content of liver from accidental death was 485 blue units (Moore) or 160 I. U. per g. of fresh liver.

3. Diseases caused depletion of Vitamin A in liver. In some cases the depletion was to such an extent that vitamin A content could not be detected with $SbCl_3$.

4. A liver from a still birth, which was examined, was devoid of any Vitamin A content.

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EFFECT OF TEMPERATURE ON THE STABILITY OF COBRA-VENOM AND CARDIOTOXIN

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(Received for publication, June 20, 1946)

Amongst the physical factors influencing the activity of an enzyme temperature plays an important role. Complete destruction of many of the enzymes is possible only when heated at a temperature of 50°C., while many others are very little affected even at a relatively high temperature like 80°C. Miyake and Ito[1] reported that Aspergillus oryzae amylase solution retains some of its activity when heated for two hours even at a temperature of boiling water. In general, there is a characteristic temperature for each and every enzyme which when heated above that limit is completely destroyed ; there is again for each such enzyme a specific temperature at which it loses one-half of its original activity. This latter has been termed by Arrhenius as the "Critical Inactivation Temperature" of the enzyme.

In this article an account has been given of the effect of variation in temperature on the activity of crude cobra-venom and that of "Cardiotoxin", the cardiac principle, isolated from crude venom by one of the authors (unpublished work), which causes the stoppage of the movements of heart.

Experimental

Equal volumes of 1 in 50 solutions of crude cobra venom and of Cardiotoxin, made in Ringer of *p*H 7.4 to 7.6 are heated separately for 30 minutes at different temperatures ranging from 60°C to 90°C These heated solutions are then quickly cooled and finally brought back to their original volumes. Isolated toad's hearts are perfused with a fixed quantity of each of these solutions, and the minimum concentration needed for systolic arrest of the heart in each case is noted. From this the p.c. of cardiac activity of each solution is calculated.

TABLE I

Effect of variation in Temperature on Cardiotoxin

No. of Expts.	Temp. at which the substance is heated for 30 minutes	Minimum conc. producing the systolic arrest of toad's heart.	Quantity of the solution perfused	Percentage of activity remaining after heating.
1.	32°C (Room temp)	1 in 450	5 cc.	100
2.	60°C	1 in 450	5	100
3.	70°C	1 in 425	5	95
4.	75°C	1 in 375	5	85
5.	80°C	1 in 300	5	67
6.	82.5°C	1 in 225	5	50
7.	85°C	1 in 110	5	24
8.	90°C	Heart not arrested by any concentration.	5	Nil

TABLE II

Effect of variation in Temperature on crude cobra venom

No. of Expts.	Temp. at which the substance is heated for 30 minutes	Minimum conc. producing the systolic arrest of toad's heart.	Quantity of the solution perfused	Percentage of activity remaining after heating.
1.	60°C	1 in 200	5 cc.	100.0
2.	70°C	1 in 200	5	100.0
3.	75°C	1 in 175	5	87.5
4.	80°C	1 in 145	5	72.5
5.	82.5°C	1 in 110	5	55.0
6.	85°C	1 in 50	5	25.0
7.	90°C	Heart not arrested by any concentration.	5	Nil

It is observed that both cobra venom and cardiotoxin retain their cardiac activity even when heated to such a high temperature as 70°C ; complete destruction of this activity occurs only at 90°C. ; between these two extremes, cardiotoxin loses one-half of its original activity at 82.5°C. and the crude venom at 83°C. Crude cobra venom is observed to be slightly more heat-stable than cardiotoxin when both are heated at the same temperature for the same period of time. This is probably due to the fact that some inert protein which acts as an inhibitor is adsorbed on cardiotoxin molecules in crude venom and thereby protects the cardiotoxin from the effect of heat.

The reversibility of the cardiac properties, previously destroyed by heat treatment, has also been investigated. For this purpose, the solutions already inactivated, partially or completely, by heating at different temperatures, are kept inside a refrigerator, maintained at 40°C for 48 hours and then the cardiac activities of these solutions are again quantitatively measured by perfusing isolated toad's hearts as before. The experimental results are given in Tables III and IV.

TABLE III

Cardiac activity of heated cardiotoxin before and after preservation in the cold.

No. of Expts.	Temp. at which the substance is heated for 30 minutes.	Percentage of cardiac activity remaining after heat-treatment and subsequent.	
		(a) Heat-treatment	(b) Preserving at 4°C for 48 hours.
1.	75°C	85.0	85.0
2.	80°C	67.0	67.0
3.	82.5°C	50.0	50.0
4.	85°C	24.0	24.0
5.	90°C	Nil.	Nil.

TABLE IV

Cardiac activity of heated cardiotoxin before and after preservation in the cold.

No. of Expts.	Temp. at which the substance is heated for 30 minutes.	Percentage of cardiac activity remaining after heat-treatment and subsequent.	
		(a) Heat-treatment	(b) Preserving at 4°C for 48 hours.
1.	75°C	87.5	86
2.	80°C	72.0	72
3.	82.5°C	55.0	55
4.	90°C	Nil	Nil

Conclusion

From the experimental results it appears that in the crude venom or in the cardiotoxin, the process of inactivation brought about by heat-treatment is irreversible ; for in either case, the lost cardiac activity cannot be restored to the inactivated solutions even by preserving in the cold for 48 hours or more.

Acknowledgement

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EFFECT OF METHYLENE BLUE ON THE TOXICITY OF COBRA VENOM

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Brooks (1) and Hajlik (2) reported that methylene blue could be effectively used against carbon monoxide and cyanide poisoning and Geiger (3) actually recorded several clinical observations where this dye had been used with satisfactory results in those cases. According to Crisler (4), methylene blue is not only useless in the treatment of such cases, but toxic in nature ; for, it increases anoxæmia in animals. On the contrary, marked rise of blood-pressure has been observed by Garfounkel and Gautrelet (5) and also by Lundberg (6). Stimulation of an exercised frog-heart has been noticed by Lundberg (6) with small doses of methylene blue.

Macht and Davis (7) studied the antagonistic action of methylene blue against venom (Naja Tripudian) on various species of animals, such as mice, rats, guinea-pigs and pigeons, and pointed out that the toxicity of the venoms was not affected much, and remained almost the same when methylene blue was administered along with the venom into an animal. The increasing importance of methylene blue in recent days as an antidote demands further investigation in this direction.

Experimental

A 0.02% solution of methylene blue in normal Ringer of pH 7.4 to 7.6 is prepared; from this stock solution, different dilutions of the dye are made with the same Ringer. Excised toad's heart is then perfused with solutions containing venom, 1 in 200 and the dye in different amounts. The results of our experiments are recorded in Table I.

For studing the effect *in vivo*, experiments are undertaken on cats. The cat, after being properly anæsthetised, is placed on the table and its carotid blood-pressure recorded. Different amount of methylene blue with a fixed amount of cobra venom (2 mgs. per kg. of body weight) are slowly administered through the femoral vein and the changes of blood-pressure following the administration of the mixture are recorded. The results are given in Table II.

TABLE I

No.	Substances with which perfused	Concentration of the substances in perfusion fluid.	Quantity perfused.	Condition of heart after perfusion.
1.	Methylene blue	1 in 50,000	5 cc.	Slightly stimulated
2.	Methylene blue	1 in 10,000	5	Normal
3.	Venom	1 in 200	5	Stopped in systole
4.	Mixed sol. containing venom and methylene blue	Venom 1 in 200 & Methylene blue 1 in 50,000	5	Stopped in systole
5.	Mixed sol. containing venom and methylene blue	Venom 1 in 200 & Methylene blue 1 in 10,000	5	Stopped in systole

TABLE II

No.	Weight of at in Kilo.	Amounts of venom & Methylene blue administered.	Effect on circulation
1.	2.2	4.5 mgs. of venom and 2.0 mgs. of methylene blue	Steep fall in blood-pressure.
2.	2.5	5.0 mgs. of venom and 3.0 mgs. of methylene blue	Steep fall in blood-pressure.
3.	2.4	4.85 mgs. of venom and 4.0 mgs. of methylene blue	Steep fall in blood-pressure.
4.	2.0	4.0 mgs. of venom and 6.0 mgs. of methylene blue	Steep fall in blood-pressure.

Conclusion

Neither the perfusion experiments on the isolated toad's heart nor the experiments on the cat *in vivo* show any antagonistic or neutralising effect of methylene blue on the toxicity of cobra venom. Similar experiments with cardiotoxin, an active principle isolated from crude venom by one of the authors (unpublished work) and responsible for the stoppage of the movements of heart, also reveal that methylene blue has no influence in altering its potency in any way.

Acknowledgement

Our best thanks are due to Dr. B. N. Ghosh, D.Sc. (Lond.), F.N.I. and Dr. B. B. Sarkar, D.Sc. for their valuable advice and keen interest during the progress of the work.

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EFFECT OF ULTRA-VIOLET RAYS ON THE STABILITY OF COBRA-VENOM AND CARDIOTOXIN

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Complete destruction of enzymatic activities brought about by ultra-violet rays has been recorded by various workers. For example, urease is completely destroyed when exposed to ultra-violet rays (Tauber, 1). Similar phenomena have also been observed by Pincussen (2) with malt diastase and by Uehra (3) with pepsin.

Massol (4) first observed that when cobra-venom is exposed to ultra-violet rays from a quartz-mercury vapour lamp it gradually loses its activity. Macht and Davis (5) also observed that a solution of cobra-venom (Naja Naja), exposed for 15 minutes to ultra-violet rays from a water-cooled quartz lamp, suffered a diminutive in its toxicity to the extent at 85% of its original value.

In the course of our studies on the effect of ultra-violet rays on the cardiac activities of the crude venom and of the cardiatoxin, isolated from it by one of the authors (unpublished work) we made use of cold quartz lamp. This lamp emits radiation of limited range of wave lengths without any appreciable production of heat and was very useful for our work. The burner consisted of a quartz tubing containing neon gas and mercury vapour through which passed an electric current under a very high voltage. Radiation from such a lamp mainly consisted of one spectral line of very high intensity at 254 mμ and this constituted 97% of the total radiation.

EXPERIMENTAL

25 Cm. of a 5.0% solution of the venom (or of the cardiotoxin), in Ringer of *p*H 7.4 to 7.6, are exposed to ultra-violet rays, placed at a distance of 20 cm. from the source of light, the duration of exposure being varied in every case. The requisite exposure having been completed each time the exposed solution is tested for its cardiac activity by the usual method of perfusion on isolated toad's heart. The results are as follows:—

TABLE I

No. of Expts.	Duration of exposure to ultra-violet rays (in minutes).	Minimum conc. producing the systolic arrest of toad's heart.	Quantity of solution perfused.	Percentage of activity remaining after exposure.
1	0	1 in 450	5 cc.	100
2	30	1 in 420	5	90
3	50	1 in 350	5	78
4	70	1 in 290	5	64
5	90	1 in 200	5	45
6	120	1 in 90	5	20
7	150	Arrest of the heart could not be produced at any conc.	5	Nil

TABLE II

Exposure of cobra-venom to ultra-violet rays.

No. of Expts.	Duration of exposure to ultra-violet rays (in minutes).	Minimum conc. producing the systolic arrest of toad's heart.	Quantity of solution perfused.	Percentage of activity remaining after exposure.
1	0	1 in 200	5 cc.	100
2	30	1 in 185	5	92
3	50	1 in 160	5	80
4	80	1 in 125	5	62.5
5	120	1 in 70	5	35
6	150	1 in 20	5	10
7	200	1 in 20 Arrest of the heart could not be produced at any conc.	5	Nil

Conclusion

It appears that the toxicity of the venom is greatly reduced when irradiated by ultra-violet rays and, the longer the time of exposure, the more pronounced is the decrease of cardiac activity of the irradiated material. Cardiotoxin, under similar circumstances, appears to lose its cardiac property more rapidly than the crude venom.

Acknowledgement

Our best thanks are due to Dr. B. N. Ghosh, D.SC. (Lond.), F.N.I., and Dr. B. B. Sarkar, D.SC., for their keen interest taken during the progress of the work.

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EFFECT OF DIFFERENT ADSORBENTS ON THE TOXICITY OF COBRA-VENOM

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Many substances, such as charcoal, kaolin etc., have the power of adsorbing various toxins and antitoxins ; tetanus toxin. for example, is most effectively adsorbed by almost all common adsorbents ; the adsorption of diptheria toxin and antitoxin on the other hand is only possible with animal charcoal (Jacque and Zunz, 1), while various alkaloids and organic bases have been found to be quite effectively adsorbed by charcoal (Loffler and Spiro, 2). It is, therefore, possible that among the common adsorbents such as charcoal, kaolin, etc., one or the other may be found efficient enough to adsorb the toxic constituents of cobra-venom. With the object in view, an attempt has been made to observe the effect of various known adsorbents on the toxicity of the crude venom and of cardiotoxin, isolated from it, by one of the authors (unpublished work.)

Crude venom is dissolved in Ringer of pH 7.4 to 7.6 and brought to a concentration of 1 in 200. Equal volumes of this solution (20 cc.) are taken in five separate clean and dry test tubes, and to each is added the adsorbent, to be tested, in different quantities. The tubes are then mechanically shaken for about 1 hour and the contents quickly filtered separately. Each filtrate is tested for its cardiac activity by the usual perfusion method on isolated toad's heart.

Three series of observations were thus taken using animal charcoal, kaolin and kieselguhr and the respective adsorbents. The results are given in Tables I, II and III. Such observations were also similarly taken using 'Cardiotoxin' in place of crude venom.

TABLE I

Effect of activated animal charcoal on the toxicity of crude venom.

No.	Vol. of the venom soln. taken.	Concentration of the venom solution.	Amount of activated charcoal added.	Condition of heart when perfusion with 5 cc. of the filtrate
1	20 cc.	1 in 200	0.2 g.	Stops in systole
2	20	1 in 200	0.5	Stops in systole
3	20	1 in 200	1.0	Stops in systole
4	20	1 in 200	1.5	Complete systolic arrest not observed.
5	20	1 in 200	2.0	Does not stop.

TABLE II

Effect of Kaolin on the toxicity of crude venom.

No.	Vol. of the venom soln. taken.	Concentration of the venom solution.	Amount of kaolin added.	Condition of heart when perfusion with 5 cc. of the filtrate
1	20 cc.	1 in 200	0.5 g.	Stops in systole
2	20	1 in 200	1.0	Stops in systole
3	20	1 in 200	1.5	Stops in systole
4	20	1 in 200	2.0	Stops in systole
5	20	1 in 200	3.0	Stops in systole

TABLE III

Effect of Kieselguhr on the toxicity of crude venom.

No.	Vol. of the venom soln. taken.	Concentration of the venom solution.	Amount of Kieselguhr added.	Condition of heart when perfusion with 5 cc. of the filtrate
1	20 cc.	1 in 200	1.0 g.	Stops in systole
2	20	1 in 200	1.5	Stops in systole
3	20	1 in 200	2.0	Stops in systole
4	20	1 in 200	2.5	Stops in systole
5	20	1 in 200	3.0	Stops in systole

Conclusion

It appears from the above results that amongst the common adsorbents, only activated animal charcoal can effectively adsorb the toxic constituents of cobra-venom. And the same appears to be true in respect of the isolated principle "Cardiotoxin" as well.

Acknowledgement

Our best thanks are due to Dr. B. N. Ghosh, D.Sc. (Lond.), F.N.I., and Dr. B. B. Sarkar, D.Sc., for their keen interest during the progress of the work.

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STUDIES ON CHOLINE-ESTERASE

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According to Michaelis, the iso-electric point is that pH at which the protein particles migrate neither to the anode nor to the cathode in an electric field. Of the different characteristics of a protein the iso-electric point is a very important one, controlling its nature and properties. Most proteins have iso-electric points within pH 5 to 7. Those with iso-electric points below pH 5 can be described as acid proteins and those with iso-electric points above pH 7.0 are basic proteins.

The conception of iso-electric point with which the physical chemistry of proteins was first begun, was introduced by Hardy (1). He observed that the protein particles behave as ions when an electric current is passed through the solution and migrate either to the anode or to the cathode according to the nature of the electrical charge they carry.

Loeb (2, 3) put forward the view that the iso-electric behaviour of a protein can be explained from the purely chemical standpoint. The proteins are amphoteric in nature and in solution react with the acid or alkali as the case may be, to form

highly dissociable salts i.e., metal acid proteinates or metal proteinates. The iso-electric point is, therefore, defined as that hydrogen ion concentration at which the protein solution is unable to form either metal proteinates or metal acid proteinates.

It is known that if a protein solution is shaken with colloidal particles, the particles absorb the protein molecules and possess the charge identical with those of the protein. Loeb determined the iso-electric point of gelatin and egg-albumin with the help of this procedure and found the result to be in good agreement with that obtained in solution of these proteins.

Abramson (4) also determined the iso-electric point of serum albumin absorbed on quartz particles by means of the micro-cataphoretic method and compared the result obtained with that of Tiselius (5) determined in a U-tube for the same protein and showed that the electric mobilities in both the cases are in excellent coincidence within the limits of experimental error. But he noticed that the iso-electric point is shifted slightly due to the absorption on the quartz particles.

Recently Mayer (6) found that the electric mobility of horse serum pseudo-globin absorbed on collodion particles is the same as that of the pure protein solution. For the determination of the iso-electric point by this procedure, the charge of the particles is determined at different pH. The pH at which the charge is neither positive nor negative is taken to be the iso-electric point of the protein in the medium in question.

The iso-electric point is also determined by the cataphoretic method in a U-tube. From the measurement of the activities associated with the protein at the two electrodes its iso-electric point is found out.

The buffer index is also utilised for the determination of the iso-electric point. The essence of this method is to dissolve the protein in buffers of different pH and the point at which the shift of pH will be at a minimum will be considered as the iso-electric point of the protein concerned.

It is also known that ions of buffer solutions used in the determination of iso-electric point may have some effect, since the charge on a colloidal particle is not only affected by the hydrogen ion concentration but also by the other ions present in the solution.

To determine the iso-electric point of choline-esterase isolated from the venoms of Naja Tripudians (7) the method utilised is that of the measurement of the charge of the quartz-coated protein particles under the microscope in a micro-cataphoretic cell. The result thus obtained is verified by measuring the cataphoretic velocity of the enzyme particles in a U-tube in an electric field.

Experimental

Micro-cataphoretic method.

A small quantity of very finely divided quartz in aqueous suspension as prescribed by Abramson[8,9] is shaken with the choline esterate for a few minutes and

the resultant solution is diluted with buffer solution so that the final solution has an ionic strength of 0.02M. The buffer solutions used in this experiment are those prepared with phosphate and phthalate according to Sorensen. The cataphoretic velocity of the quartz-coated protein particles is measured at different pH in a flat-rectangular cell of the Abramson (8) type. The pH of the different solutions is verified by measurement with glass electrode and the specific conductance of each solution is measured by the Wheatstone bridge. The iso-electric point of choline-esterase, determined by this method, is at pH 5.85 as shown in Table I. The positive and negative signs indicate the nature of the charge carried by the particles.

TABLE I

pH.	μ/cm./volt/sec.
4.20	1.12×10^{-5}
4.80	0.60×10^{-5}
5.00	0.47×10^{-5}
5.50	0.21×10^{-5}
5.85	0.00×10^{-5}
6.00	-0.06×10^{-5}
6.50	-0.20×10^{-5}
7.00	-0.45×10^{-5}
8.00	-0.76×10^{-5}

Cataphoretic method.

The apparatus used in this method is a U-tube with two stop-cocks fitted in its two limbs at the middle. A solution of choline esterase of known strength is mixed with 1 cc. of buffer solution and made up to a total volume of 10 cc. so that the final concentration of this enzyme is 0.2%. The buffer solutions used are prepared from phosphate and phthalate as prescribed by Sorensen. The stop-cock at the bottom of the side limb is opened and the buffered protein solution poured with care into the tube from the funnel at the top of the side limb so that the holes of the stop-cocks of the U-tube are also filled up. The buffers diluted in the proportion given above are poured to fill the upper parts of the U-tube. The solutions in the upper parts are connected with copper sulphate solution in two glass beakers through agar bridge tubes. The agar bridges bent in the form of U are filled with 3% agar agar in 0.85% sodium chloride. The positive end from the main of 220 volt is connected to the beaker containing copper sulphate solution through a milliammeter and a copper electrode and the negative through a key and a copper electrode. The current is then allowed to pass through the whole column of the liquid in the U-tube for a definite interval of time.

As there is the possibility of a slight rise of temperature during the passage of current, the U-tube is immersed in a bath and the temperature maintained constantly adding ice-cold water as necessity demands. After the requisite period the solutions from both the limbs are separately withdrawn and then choline esterase activity determined. The results are tabulated below.

TABLE II

Electrophoresis of choline-esterase solution.

Phthalate buffer.

pH.	Unit of choline esterase.	
	Anode.	Cathode.
6.30	43	0
6.00	23	7
5.95	16	10
5.90	1	1
5.85	10	15
5.80	8	24

Phosphate buffer.

pH	Unit of choline esterase	
	Anode	Cathode
6.30	44	0
6.00	24	7
5.95	15	10
5.90	1	1
5.85	10	16
5.80	7	25

Summary and Discussion

The iso-electric point of pure choline-esterase as obtained by the two methods mentioned above differs from each other within the limit of experimental error. One method gives pH 5.55 while the other gives pH 5.9. This slight shift is quite in agreement with the findings of Abramson[8] while determining the iso-electric point of serum albumin. This small deviation may be due to the protein particle being absorbed on the surface of the quartz suspension resulting in the slight change in the electrical behaviour. The iso-electric point in the two buffers is also found to be in good agreement indicating thereby that the different ions present in two buffers have got no appreciable influence on the iso-electric point of choline-esterase under the conditions defined above.

The author expresses his gratitude to Dr. B. N. Ghosh, D.Sc. (Lond.) for his helpful suggestions during the progress of the work and also to the authorities of the Calcutta University for granting a research scholarship from the grant of the Bengal Immunity Co., Ltd.

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ADVANCEMENT OF KNOWLEDGE SERVICE TO MANKIND

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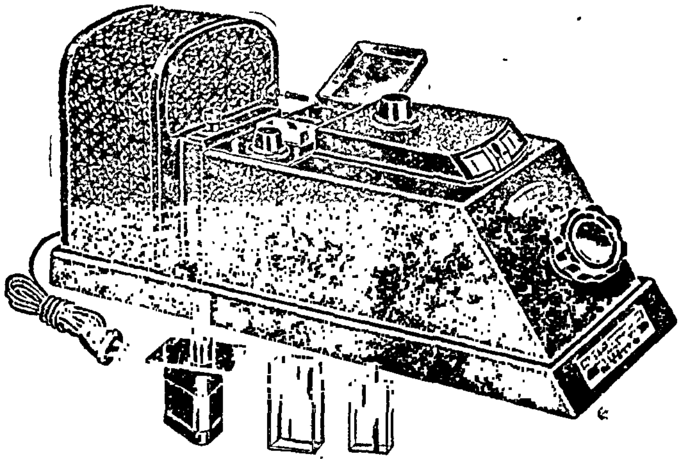

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CONSTITUTION OF COUMARINS ISOLATED FROM *FERULA ALLIACEA*.

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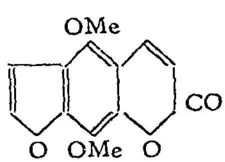
(Received for publication, January 28, 1946)

While investigating the oil from the fruits of *Ferula alliacea*, Boiss. (N. O. *Umbelliferae*), Bose and Dutt (1) observed that a petroleum ether extract of the crushed fruits deposited a yellow solid product on standing. A detailed examination proved this to be a mixture, from which the authors were finally able to isolate *iso*pimpinellin (I) and another substance, m.p. 88°, having the formula, $C_{17}H_{16}O_6$. As its properties did not agree with those of any known compound, it was thought to be a new compound and accordingly named 'Ferulin'.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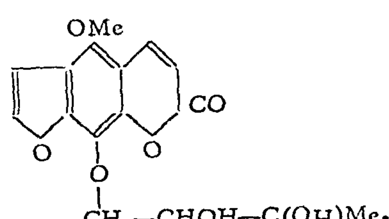

(II)

The combined quantities of ferulin and *iso*pimpinellin however accounted for a fraction of the total solid. The present investigation was undertaken with the object of (i) isolating other constituents of the mixture, if possible, and (ii) elucidating the structure of ferulin by a more detailed study of its reactions and degradation products. As the solid deposit mentioned above formed the starting material of the present work, endeavours were at first made to obtain a better yield than that recorded by Dutt (2), who, using petroleum ether as the solvent, obtained a yield of 1.2 per cent.

Modified Method of Extraction

The solid matters could be easily extracted from the crushed fruits by ether, benzene, chloroform and alcohol. Of these, ether gave the best yield, namely 1.7 per cent. The yellow solid melted at 78-80°. It is insoluble in petroleum ether, sparingly soluble in ether and water and readily soluble in choroform, benzene, ethyl acetate, warm alcohol, acetone and pyridine.

Purified by repeated crystallisations from different solvents or their mixtures under varying experimental conditions, the crude product formed yellow opaque nodules or discs having sometimes a crystalline edge. The m.p. varied between 84° and 87°. Its behaviour and appearance under the microscope led us to suspect that it was a mixture—a supposition which has subsequently been confirmed by the isolation of definite products. It was free from sulphur, halogen and nitrogen. It was neutral to litmus and indifferent to ferric ion in alcohol. Although insoluble in aqueous sodium carbonate or 1% aqueous alkali, it readily dissolved in aqueous alcoholic alkali with a brown colour, from which no precipitate was obtained on dilution with water. This behaviour is compatible with the lactonic character of the crude product which, for the sake of convenience, will henceforth be termed "mixed lactone".

Effect of Steam on the Ether Extract

It has been reported that the fruits of *F. alliacea* contain about 0.9% essential oil (Bose and Dutt, 1). Essential oils have often a pronounced solvent action on many organic compounds, and it was believed that the removal of the essential oil from the extract might enhance the yield of "mixed lactone". Accordingly an ether extract of crushed seeds of *F. alliacea* was distilled in steam. Eventually a pale yellow crystalline solid, m.p. 123-25° (dry) and *iso*pimpinellin (I) were isolated from the non-volatile residue (*vide* Experimental).

The former had properties similar to those of "mixed lactone". It was soluble in hot water, acetone, chloroform, pyridine, hot benzene and hot ethyl acetate, and almost insoluble in ether and petroleum ether. The yield was 3.2% (crude) or a little over 1.5% (pure) based on the weight of seeds. The analytical data indicated the composition $C_{16}H_{15}O_6(OCH_3)$ after drying in vacuo.

Application of Spaeth's Method

Having been unable to isolate "mixed lactone" by the above method, Späth's method for the isolation of coumarins was attempted. It is a matter of general experience that natural coumarins do not always separate out spontaneously from fatty or essential oils. Recently Späth (3) described a method for the isolation of natural coumarins when present in oils in solution. This method was applied to the clear oil left after separation of the "mixed lactone" from the ether extract, when an acid, m.p. 194-5° decomp. was finally obtained. This will be called

'Ferulinic Acid'. Neither the "mixed lactone," nor the compound, m.p. 123.25° could be obtained by this method.

Ferulinic acid forms pale brown prisms, sparingly soluble in hot water, but easily in most organic solvents excluding ether and petroleum ether. It is acidic towards litmus and decomposes sodium bicarbonate solution. It is indifferent towards ferric chloride. It decomposes partially when distilled in high vacuum, but does not produce any neutral substance by loss of water. Boiling acetic anhydride and fused sodium acetate produced a gummy acid which could not be crystallised. Ferulinic acid analyses for $C_{15}H_{14}O_4(OCH_3).CO_2H$, and is isomeric with the compound, m.p. 123-25°. It is unsaturated and absorbs two atoms of hydrogen in presence of palladium catalyst forming a dihydro-compound, m.p. 167°.

The results so far obtained may therefore be briefly summarised thus:

(*a*) An ether extract of the fruits of *F. alliacea* deposits spontaneously on standing, a yellow, neutral, non-crystallisable solid product, m.p. 78-80° (purified, m.p. 84-87°), which appears to be a mixture ("mixed lactone").

(*b*) Steam distillation of the ether extract gives *iso*pimpinellin (I) and a neutral, crystalline compound, $C_{16}H_{15}O_6(OCH_3)$, m.p. 123-5° (anhydrous) different from "mixed lactone" mentioned under (*a*) but isomeric with the acid described under (*c*).

(*c*) The ether extract after separation of "mixed lactone", gives a crystalline acid, $C_{17}H_{18}O_7$, m.p. 194-5°d. on treatment with alkali.

Various questions now presented themselves:

(*i*) Is the compound, m.p. 123-25°, a component of the "mixed lactone" or is it a transformation product of one of the components of the "mixed lactone", under the influence of steam?

(*ii*) Is ferulinic acid derived from one of the components of the "mixed lactone" or is it derived from some other constituents of the oil, or is it present as such in the oil?

(*iii*) What are the relations between the three products and how can the formation of the compound, m.p. 123-25°, and its isomer, ferulinic acid be explained?

In order to throw some light on these problems, the following experiments were performed.

(1) A sample of "mixed lactone" was boiled with water during four hours in presence of a little oxalic acid and from the resulting product the compound, m p. 123-25°, was finally isolated. Moreover the fact that the "mixed lactone" is highly soluble in ethyl acetate and benzene whereas the compound, m.p. 123-25°, is only

sparingly so, definitely ruled out the existence of the latter in the "mixed lectone". The compound, m.p. 123-25°, must therefore have been derived from one of the components of the "mixed lactone" under the experimental conditions. The other product was *iso*pimpinellin.

(2) Secondly, it was observed that the "mixed lactone", on short treatment with alcoholic alkali, gave ferulinic acid and another neutral compound identified as *iso*pimpinellin. . The "mixed lactone" therefore must have contained *iso*-pimpinellin and at least one other component which furnished ferulinic acid. It was moreover observed that neither the compound, m.p. 123-25°, nor *iso*pimpinellin, nor the products of hydrolysis (of the "mixed lactone") could give rise to this acid by alkali treatment. It may therefore be argued that both the compound, m.p. 123-25°, and ferulinic acid must have been derived from the identical component of the "mixed lactone".

(3) The next point which engaged the authors' attention was the nature of the particular constituent of "mixed lactone" which is the precursor of ferulinic acid and its isomer. It has already been reported that ferulin is one of the components of the "mixed lactone". The fact that the molecular formula of ferulin differs from these two compounds by H_2O suggests that they are probably derived from ferulin by hydrolysis. To settle this question ferulin was isolated from the "mixed lactone" by a tedious process of repeated fractional distillation in high vacuum followed by crystallisations from suitable solvents. Ferulin thus obtained in a yield of 0.5% gave the compound, m.p. 123-25°, when heated with acidulated water and was also quantitatively converted into ferulinic acid by the action of alkali. Ferulin is therefore the precursor of the neutral compound, m.p. 123-25°, and of ferulinic acid. The relationship between the three compounds is shown thus:

"Mixed lactone" ⟶ *iso*Pimpinellin, $C_{13}H_{10}O_5$

↓

Ferulin, $C_{17}H_{16}O_6$ $\xrightarrow[\text{(alkali)}]{+H_2O}$ Ferulinic acid, $C_{17}H_{18}O_7$

↓ $+H_2O$ (acid)

Compound, $C_{17}H_{18}O_7$,
m.p. 123-25°, dry
(Ferulin hydrate).

The Constitution of Ferulin

An account of the attempts made to elucidate the constitution of ferulin will now be presented. It produced a diacetyl derivative identical with that obtained from its hydrate. Deacetylation furnished ferulin hydrate, i.e., the compound, m.p 123-25°. The presence of CO-group in ferulin could not be established.

Two coumarins having the same compositions have been recently described by Noguchi and Kawanami (4, 5) who isolated them from the roots of *Angelica*

glabra, Makino (N.O. *Umbelliferae*) and named them byak-angelicin (II) and byak-angelicol (III) respectively. A comparison of the properties of byak-angelicin with those of the compound, m.p. 123-25° indicated many points of similarity as will be evident from the following table:

TABLE I.

	Byak-angelicin	*Ferulin hydrate*
Mol. Formula..................	$C_{17}H_{18}O_7$	$C_{17}H_{18}O_7$
Color and cryst. form......	Pale yellow needles.	Pale yellow flattened needles or elongated plates.
M.p..............................	117-18° (hydrated) 125-26° (anhydrous)	From 116° (variable) 118-22° (air-dried) 123-25° (anhydrous) 126-28° (,,)*
Solubility........................	Insol: Ether and Pet. ether. Sol. with difficulty: benzene, ethyl acetate. Easily sol: acetone, chloroform, pyridine, alcohol, hot water.	Same.
Alkali.............................	Soluble, pptd. unchanged by acids.	Yellow solution ; pptd. unchanged by acids.
Removal of H_2O of crystallisation.....................	By heating at 110° in vacuo over P_2O_5 for 7 hrs.	By heating in vacuo at 100° for 8-10 hrs. over P_2O_5: organic solvents also tenaciously retained.
$[\alpha]^d$ in pyridine...........	+21.62° at 25°	+25.26° at 30°.
Diacetate.........................	Pale yellow plates, m.p. 118-19°.	Colourless needles, m.p. 113°.
Chronic acid oxidation products........	(*a*) Acetone. (*b*) Bergaptene quinone.	(*a*) Same. (*b*) Same.

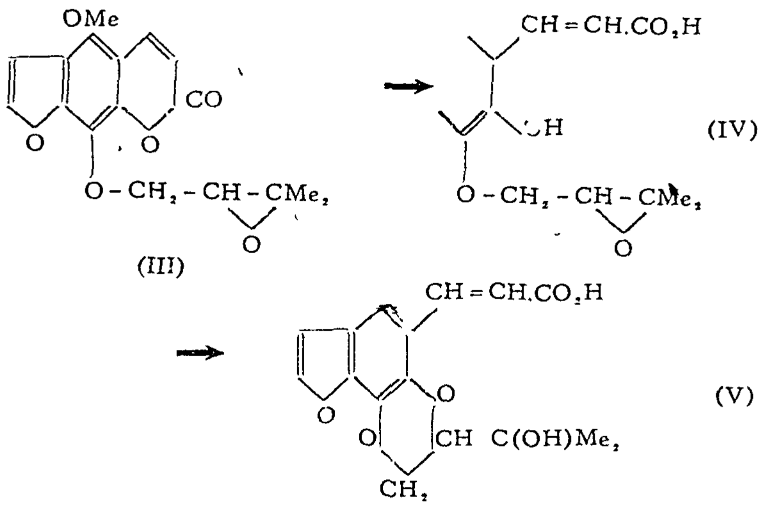
*Specimen after distillation in high vacuum and subsequent crystallisations.

A direct comparison of byak-angelicin and its acetate with the corresponding products prepared by us was rendered possible through the kindness of Professor T. Noguchi, to whom our best thanks are due. The results which are indicated in Table II definitely establish the identity of our compound (m.p. 123-25°; ferulin hydrate) with byak-angelicin (II).

TABLE II.

Byak-angelicin	*Ferulin hydrate*
1. Cream coloured (anhydrous).	Pale creamy yellow (anhydrous).
2. M.p. after cryst. from water and drying to const. weight in vacuo: shrinks at 121° and melts at 122-4°.	M.p. 123-5° (anhydrous) similarly prepared.
Mixed m.p.: shrinks at 121° and melts at 122-4°.	
3. Needles under microscope.	Flattened needles.
4. Diacetate, pale yellow thick, crystals, m.p. 115-16°. Mixed m.p. 112-16°.	Diacetate, colorless needles, m.p. 112-13°.

The formation of byak-angelicin (II) from ferulin is obviously analogous to that of oxypeucedanin hydrate from oxypeucedanin (Späth and Klager, 6). Ferulin consequently should possess a dihydroisoprene oxide chain as in oxypeucedanin and should have the structure (III) which has been advanced by Noguchi and co-workers for byak-angelicol. On the basis of formula III, the formation of ferulinic acid (V) should be expected to proceed through the intermediate compound (IV).



The physical and chemical properties of byak-angelicol and ferulin are recorded in Table III. It is evident that the divergence is too wide to suggest their identity. On the other hand ferulin does not admit of any other basic structure, in view of what has been stated above. This anomaly can only be settled by a direct comparison and detailed study of the two compounds and a further communication will be made when such an opportunity arises.

TABLE III.

Byak angelicol	*Ferulin*
1. Pale yellow plates, m.p. 106°.	Colorless transparent plates, m.p. 87°.
2. $[\alpha]^{d}23° = +34.77°$ (pyridine).	$[\alpha]^{d}23° = +27.31°$ (acetone).
3. *Sparingly* soluble in acetone, benzene and *hot* ethyl acetate.	Readily soluble *in cold* in the three solvents.
4. Byak-angelicol+KOH ——→ *iso*byakangelicol acid, white rhombic plates, m.p. 220°, *without* decomposition.	Ferulin+KOH ——→ Ferulinic acid, pale brown prisms, m.p. 194-5° *with* decomposition.
5. Acetyl derivative of the above acid, m.p. 200°.	No crystalline product.
6. Dihydro-derivative of the acid, needles, m.p. 152°.	Dihydroferulinic acid, colorless flattened needles, m.p. 167°.

Incidentally it may be noted that Späth's method (3) of isolation of coumarins will not be applicable to compounds having a dihydroisoprene oxide residue attached to position 8, either directly or through an oxygen atom.

EXPERIMENTAL

Isolation of "mixed lactone".—The fruits of *F. alliacea* were crushed to powder and extracted with ether in a Soxhlet apparatus during 80 hours. Ether was then removed from the extract on the water-bath and finally under diminished pressure. The dark greenish residue was allowed to stand at room temperature for about a week and the semi-crystalline yellow product, which had separated out, was filtered off under suction and washed with petroleum ether and a little ether. Yield 1.7 per cent. Recrystallised from methyl alcohol it formed opaque nodules or discs, m.p. 84° to 87°.

Isolation of Byak-angelicin and Essential Oil.—Another specimen of the above ether extract, after removal of ether, was distilled in steam for 7 hours. The distillate was extracted with ether, dried over sodium sulphate and ether removed under reduced pressure at 40-50°. A pale yellow pleasant-smelling oil (0.2%) was left. The yield of the oil varies considerably with the quality of the raw material. It was found to possess the following constants:

Density at 33°	0.8695
Refractive index at 31°	1.4640

The oil is optically inactive and is non-phenolic in nature.

The viscous oily product, not volatile in steam, was allowed to stand at room temperature for a few days. Greenish yellow crystals, which had separated out, were collected and washed with petroleum ether and ether to remove the greater portion of the adhering oil. The crude product amounted to 3.2%. It was successively crystallised from 95% alcohol, ethyl acetate, 25% alcohol and finally from hot water. The pale yellow, long, flattened needles melted at 118-22° after drying in air. Further crystallisations did not materially alter the m.p.

I. A specimen (crystallised from benzene) weighing 350.4 mg. lost 25.3 mg. on drying at 95° for 5 hours in vacuo over P_2O_5: Hence volatile matters lost=7.22%. M.p. after drying, 123-25°.

II. A specimen, m.p. 119-22° (vacuum distilled and crystallised from ethyl acetate) weighing 438.8 mg. lost 40.3 mg. on drying to constant weight at 100° for 8 hrs. in vacuo over P_2O_5: Hence volatile matters lost=9.19%. M.p. after drying, 126-28°.

The colour of the samples became lighter after drying, but the crystals did not crumble to powder, which happens to be usually the case when solvents of crystallisation are present. The presence of solvents of crystallisation appears doubtful, but there is no doubt that they are tenaciously retained.

Found (dry sample): C, 61.36; H, 5.3; OMe, 8.8. Calc. for $C_{17}H_{18}O_7$: C, 61.08 ; H, 5.4 ; OMe, 9.28 per cent.

A specimen of fairly pure byak-angelicin (from *F. alliacea*) was distilled in high vacuum (210-220°/0.08 mm.), and crystallised from alcohol (m.p. 117-25°) and then from benzene (m.p. 115-24°). Dried to constant weight in vacuo over P_2O_5 at 100°, it melted at 126-8°.

[Found (dry sample): C, 61.25; H, 5.6. Calc. for $C_{17}H_{18}O_7$: C, 61.08; H, 5.4 per cent.]

0.2771 g. dry byak-angelicin (from *F. alliacea*) dissolved in 25 cc. pyridine showed a rotation of +0.28° in 1 dcm. tube at 30°. Hence $[\alpha]^{d} = +25.26°$.

Isolation of isopimpinellin.—A specimen of crude byak-angelicin (from *F. alliacea*), which had been only twice crystallised from dilute methyl alcohol, was distilled in vacuum. Two fractions were collected. (i) B.p. below 180°/0.06 mm., and (ii) b.p. 210-220°/0.06 mm. Fraction (i) was crystallised from alcohol, redistilled in high vacuum and then repeatedly crystallised from alcohol till the m.p. became constant at 148-50°. Mixed with an authentic specimen of *iso*pimpinellin there was no depression in m.p.

Fraction (*ii*) on repeated crystallisations from hot water gave byak-angelicin, m.p. 119-22° (air dried).

Diacetylbyak-angelicin.—Byak-angelicin (0.507 g. from *F. alliacea*), acetic anhydride (10 cc.) and fused sodium acetate (4 g.) were gently boiled for 4 hours and then poured into cold water. The precipitate was collected, and crystallised from dilute methyl alcohol (charcoal), when colorless needles, m.p. 112-13°, were obtained. (Found in dry specimen: OMe, 7.44. Calc. for $C_{21}H_{23}O_9$: OMe, 7.2 per cent).

Deacetylation of the Above Compound.—The diacetate (1 part) dissolved in alcohol was heated with sodium hydroxide (1 part) for 15 minutes on the water-bath. The deep yellow solution was cooled and acidified with hydrochloric acid. The pale yellow needles melted at 122-23° (ethyl acetate). No depression in m.p. was noticed when mixed with byak-angelicin having almost the same m.p.

Oxidation of Byak-angelicin (from F. alliacea) with Chromic Acid: Isolation of (a) Bergaptene quinone and identification of (b) Acetone.

(*a*) Byak-angelicin (1g.), dissolved in glacial acetic acid (10 cc.) was treated with a mixture of chromic acid (0.5 g.), water (5 cc.) and glacial acetic acid (10 cc.). The mixture was allowed to stand at room temperature for two days. The brown-red crystals, which had separated out, were collected, washed with water and dried. The product was sublimed in high vacuum (175-90°/0.05 mm.) as orange-red crystals, m.p. 250-52° decomp. (Found: C, 61.3; H, 2.18. Calc. for $C_{11}H_4O_5$: C, 61.11 ; H, 1.85 per cent.). Bergaptene quinone melts at 250°decomp. (7).

(*b*) The acetic acid filtrate, left after the separation of bergaptene quinone, was distilled and about 15 cc. of the distillate were collected. This was made alkaline and redistilled. The distillate (10 cc.) was treated with 2 drops of benzaldehyde (pure) and 2 drops of 10% sodium hydroxide. Sufficient alcohol was then added to obtain a clear solution, which was allowed to stand for about a week, in a closed vessel. Pale yellow flakes gradually separated out. The crystals were collected and recrystallised from dilute alcohol several times when pale yellow plates, m.p. 109-10°, were obtained. It did not depress the m.p. (108-10°) of dibenzalacetone, other physical properties of which it also shared.

Isolation of Ferulinic Acid.—The oil (from 825 g. fruits) left after the separation of the "mixed lactone" was diluted with 250 cc. methyl alcohol and treated gradually with 25 g. potassium hydroxide dissolved in 25 cc. water at 15-20°.

After allowing the mixture to stand for one hour at 20°, it was poured into 1 litre ice-cold water and was extracted with ether. The aqueous portion was acidified with hydrochloric acid (Congo-red) when a dark brown tarry product separated out. This was collected next day and spread over a porous plate to remove adhering oily impurities. The dark brown crystals thus obtained were washed with 10 cc. 80% methyl alcohol, and finally crystallised from aqueous alcohol (yield 0.5% of the fruits taken). The crude acid was dissolved in warm potassium bicarbonate solution, filtered and the clear filtrate acidified. The crystalline precipitate was recrystallised from hot water containing a little alcohol as pale brown prisms, m.p. 194-5° decomp. (Found: C, 60.87 ; H, 5.66 ; OMe 9.38. $C_{17}H_{16}O_7$ requires C, 61.08; H, 5.4; OMe, 9.28 per cent.).

Action of Oxalic Acid on Ferulinic Acid.—The acid (0.2 g.) was boiled with a dilute solution of oxalic acid (0.35 g. in 25 cc. water) for 3 hours. The crystals obtained on cooling the solution were repeatedly crystallised from hot water when pale brown prismatic crystals, m.p. 193-4° decomp. were obtained. No depression in m.p. in admixture with the parent substance was observed.

Catalytic Reduction of Ferulinic Acid.—Ferulinic acid (0.2517 g.) was reduced with palladium-charcoal (0.2 g. of 8%), which had been previously saturated with hydrogen, in alcoholic medium. 16.4 cc. hydrogen at 28° and 760 mm. were absorbed in course of 45 minutes, when further absorption practically stopped. Amount calculated for one double bond is 14 cc. The solution was freed from the catalyst, concentrated and diluted with water when dihydroferulinic acid separated out. Recrystallised from dilute alcohol, it formed colourless needles or rods, m.p. 167°. (Found: C, 60.7 ; H, 6.8. $C_{17}H_{20}O_7$ requires C, 60.72 ; H, 5.95 per cent.).

Byak-angelicin from "mixed lactone".—"Mixed lactone" (0.5 g. in 5 cc. rectified spirit) was boiled with aqueous oxalic acid (1%) for 4 hours under reflux. The yellow crystalline product obtained on cooling the solution was successively crystallised from ethyl acetate, benzene, and finally from dilute alcohol. Pale yellow crystals, m.p. 119-22°, were obtained. Its properties agreed with those of byak-angelicin. The two had almost the same mixed m.p.

Ferulinic Acid and iso*Pimpinellin from "mixed lactone".*—"Mixed lactone" (0.5 g.) was dissolved in rectified spirit (10 cc.) and treated with aqueous caustic potash (5 cc. of 10%). The mixture was evaporated on the water-bath to a small volume and acidified with hydrochloric acid. The precipitate was collected next day. This was extracted with warm potassium bicarbonate solution and filtered. The insoluble residue on repeated crystallisations from dilute alcohol gave pale yellow needles, m.p. 148°, identified as those of *iso*pimpinellin (mixed m.p.).

The bicarbonate solution on acidification with dilute hydrochloric acid gave a precipitate which formed pale brown prisms, m.p. 193-4° decomp. after repeated crystallisations from dilute alcohol. This was identified as ferulinic acid (mixed m.p.).

Isolation of Ferulin and iso*Pimpinellin from "mixed lactone".*—"Mixed lactone" (2.5 g.) was carefully fractionated in high vacuum at 0.05 mm. pressure.

The fraction which distilled below 165° was collected (1 g.). The brown residue left in the distillation tube was taken up with methyl alcohol, and allowed to crystallise slowly. The brown thick crystals thus obtained were distilled at 175-85°/0.05 mm. The pale yellow distallate (0.73 g.) on being crystallised from methyl alcohol, formed pale yellow prisms. Redistilled in high vacuum and twice crystallised from the same solvent, transparent colorless plates, m.p. 87°, were obtained. These are sparingly soluble in ether and petroleum ether, easily soluble in cold benzene, cold ethyl acetate, cold acetone and warm alcohols.

0.3606 g. substance dissolved in 25 cc. acetone showed a rotation of +0.197° in a 0.5 dcm. tube at 23°. Hence $[\alpha]^d = +27.31°$. This compound was found to be identical in every respect with ferulin, $C_{17}H_{16}O_6$, previously described by Dutt (2).

The fraction boiling below 165° was fractionally crystallised from dilute methyl alcohol. Three fractions were obtained. (i) Pale yellow ill-defined crystals, m.p. 138°: (ii) Yellow needles, m.p. 96-97°: and (iii) Pale yellow ill-defined crystals or nodules, m.p. 79-81° (extended). Fraction (i) after several crystallisations gave soft yellow needles, m.p. 148°, identified as *iso*pimpinellin (mixed m.p.). Fraction (ii) on further crystallisations formed yellow needles, m.p. 100-1°. The analytical data of this compound, which was at first thought to be a new component, were as follows:

C, 62.82; H, 4.18; OMe, 19.32.

This product on vacuum distillation gave no single product but fractions, which when crystallised from dilute alcohol, had m.p.'s 137°, 144° and below 100° (not sharp). Apparently the crystalline product, m.p. 100-1° from fraction (ii), is a mixture. As we had a small quantity of this substance at our disposal, further examination was not possible.

Conversion of Ferulin into Byak-angelicin and Ferulinic Acid.—The conversion of ferulin into byak-angelicin was carried out in the manner described before. The identity of the product with byak-angelicin was established in the usual manner.

The acid obtained by treating ferulin (0.1 g.) with 4 cc. of 10% alkali in aqueous alcoholic medium was found to melt at 194° decomp. It was identified as ferulinic acid.

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PROTEOLYTIC SYSTEM IN THE BLOOD OF TUBERCULOSIS AND TYPHOID PATIENTS

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Iyengar *et al* (1) have studied the proteolytic system in normal and pathological conditions like cancer, diabetes, typhoid, anæmia, thrombocytopænic purpura, small-pox, cholera, etc. Changes in the free trypsin content of plasma were observed in certain pathological conditions. Greater protein catabolism due not only to an active increased energy requirements, but also due to an active destruction of protein in typhoid fever and febrile tuberculosis have been reported by Krause (2). In view of this, a study of the proteolytic system of blood in these two conditions would be exceedingly interesting. Although the blood of a few typhoid patients have already been tested, it is considered that they were too few in number to come to any definite conclusions. Moreover the technique of the determination of the tryptic activity of plasma has been altered.

EXPERIMENTAL

Materials and Methods

In previous papers by the author, plasma was precipitated by acetone, the precipitate containing the trypsin centrifuged, dried in vacuum free of acetone. Instead of this, the blood after withdrawal aseptically from the patient is centrifuged and the plasma thus obtained was incubated at 37°C for 24 hours under sterile conditions. 2 cc. of this plasma were withdrawn aseptically before incubation and the non-protein nitrogen content of this determined in the usual manner after precipitating the proteins with 10% trichloracetic acid. Non-protein nitrogen was likewise determined on the sterile plasma after incubation for 24 hours. The increase in the non-protein nitrogen after incubation represented the proteolytic activity of the plasma.

The proteolytic activity of the plasma of about 20 normal human subjects were first determined.

TABLE I

Proteolytic activity of plasma from normal human subjects.

	Proteolytic activity of plasma expressed as 'Increase in N. P. N.' for 100 c.c.
(1)	18 mg.
(2)	22 mg.
(3)	25 mg.
(4)	17 mg.
(5)	24 mg.
(6)	16 mg.
(7)	28 mg.
(8)	21 mg.
(9)	15 mg.
(10)	14 mg.
(11)	26 mg.
(12)	20 mg.
(13)	25 mg.
(14)	19 mg.
(15)	21 mg.
(16)	16 mg.
(17)	14 mg.
(18)	21 mg.
(19)	18 mg.
(20)	15 mg.
Mean value	19.8 mg. or 20 mg. correct to a mg.

Standard error is $\sqrt{\left(\frac{Ed^2}{n(n-1}\right)} = \pm 1$ Approximately.

TABLE II

Proteolytic activity of plasma from patients suffering from Tuberculosis.

	Proteolytic activity of plasma expressed as 'Increase in N. P. N.' for 100 c.c.
(1)	35 mg.
(2)	31 mg.
(3)	22 mg.
(4)	39 mg.
(5)	19 mg
(6)	42 mg.
(7)	25 mg.
(8)	38 mg.
(9)	34 mg.
(10)	44 mg.
(11)	32 mg.
(12)	45 mg.
(13)	28 mg.
(14)	26 mg.
(15)	36 mg.
(16)	29 mg.
(17)	34 mg
(18)	19 mg.
(19)	25 mg.
(20)	31 mg.
Mean value	32 mg.
Standard error is	± 3

TABLE III

Proteolytic activity of plasma from patients suffring from Typhoid.

	Proteolytic activity expressed as 'Increase in N.P.N.' for 100 cc.
(1)	24 mg.
(2)	31 mg.
(3)	30 mg.
(4)	22 mg.
(5)	25 mg.
(6)	26 mg.
(7)	28 mg.
(8)	19 mg.
(9)	31 mg.
(10)	18 mg.
(11)	32 mg.
(12)	36 mg.
(13)	27 mg.
(14)	37 mg.
(15)	32 mg.
(16)	29 mg.
(17)	31 mg.
(18)	22 mg.
(19)	19 mg.
(20)	31 mg.
Mean value	28 mg.
Standard error is	± 1.5

The above results demonstrate an increase in the plasma trypsin content of Tuberculosis and Typhoid patients over those of normal subjects. This fact becomes clearer on statistical treatment of results of application of 'Significance Test.'

Significance Test (between Tables 1 and 2).

To determine whether the result in Table 1 is significantly different from the result in Table 2 or whether the difference between them is due to error of sampling, the following formula is used.

$$\frac{m_1 - m_2}{\sqrt{E_1{}^2 \times E_2{}^2}}$$ where m_1 and m_2 are the mean results and E_1 and E_2 are their respective errors.

$m_1 = 20$ $\quad E_1 = 1$

$m = {}_2 32$ $\quad E_2 = 3$

$$T = \frac{12}{\sqrt{1+9}} = 3.16.$$ P is less than 0.01

The difference in the plasma trypsin content of normal and tubercular subjects is highly significant. It might be concluded, therefore, that the trypsin content of the plasma is definitely increased in tuberculosis.

Significance Test (between Tables I and III).

$m_1 = 20 \qquad E_1 = 1$

$m_2 = 28 \qquad E_2 = 1{\cdot}5$

$T = \frac{8}{\cdot 1{\cdot}8} = 4.4.$ P is less than 0.01

This difference is also highly significant. The trypsin content of the plasma is increased in Typhoid also.

Having demonstrated that a definite increase in the trypsin content of Plasma takes place, the cause of this is next investigated. The observed increase may be due to one or other or both the following factors.

(1) The trypsin inhibitor normally present in Plasma might not be present in the blood of tubercular and typhoid patients.

(2) There might have been a real increase in the trypsin content.

In order to test the two possibilities, the following experiments were carried out.

The free trypsin content of plasma was first determined on the powder obtained from the precipitation of the plasma by acetone. The method has been described by the author (3). The trypsin content of the same plasma after precipitation by 11 volumes of 2.5% trichloracetic acid was also determined.

The results are given in Tables IV and V.

TABLE IV

Trypsin content of tubercular plasma before and after removal of trypsin inhibitor.

	Free Plasma Trypsin calculated for 100 c.c. expressed as 'Increase in N.P.N.' before removal of inhibitor.	Total Plasma Trypsin calculated for 100 c.c. expressed as 'Increase in N.P.N.' after removal of inhibitor.
(1)	23 mg.	26 mg.
(2)	25	30
(3)	28	29
(4)	22	20
(5)	35	29
(6)	34	38
(7)	26	31
(8)	31	35
Mean	28	30

TABLE V

Trypsin content of typhoid plasma before and after removal of trypsin inhibitor.

	Free Plasma Trypsin.	Total Plasma Trypsin.
(1)	31 mg.	44 mg.
(2)	24	48
(3)	35	39
(4)	26	42
(5)	38	49
(6)	31	54
(7)	27	48
(8)	32	36
Mean	31	45

In Tuberculosis the trypsin inhibitor appears to have been destroyed as there is no significant increase in the trypsin content after treatment with trichlor-acetic acid which is designed to remove the inhibitor if present. The observed increase in the plasma trypsin content in tuberculosis can be wholly attributed to the absence of the inhibitor present in normal plasma. In Typhoid the inhibitor is present, since an increase in the trypsin content is observed after treatment with trichlor-acetic acid. In this case the increase in the free plasma trypsin content is due to a real increase in the trypsin circulating in plasma.

The disappearance of the trypsin inhibitor from the plasma in tuberculosis patients is a very significant finding and the mechanism by which this happens is worth investigating. The trypsin inhibitor might not be formed at all or might have been destroyed. The only plausible way by which it could have been destroyed is by the action of a poly-peptidase which might be present in the tubercular blood, since the inhibitor is known to be a poly-peptide of low molecular weight and accordingly can be acted upon by a polypeptidase. This can be ascertained by incubating the inhibitor with tubercular plasma.

The inhibitor was prepared according to the method of Schmitz as briefly described by the author (4). A dilute solution of this (2 mg. in 25 cc.) was found to be strongly inhibiting the action of trypsin. 5 cc. of this solution was then incubated for a period of two hours with 5 cc. of tubercular plasma. The inhibitory activity of this incubated mixture on the activity of trypsin (B.D.H.) was then studied. The results are given in Table 6.

TABLE VI

	Activity expressed as increased in N.P.N. of 5 cc. of a 2% Casein solution after incubation for 6 hours.
Activity of Trypsin.	25 mg.
Trypsin plus 2.5 cc. of inhibitor (added to substrate solution after 30 minutes)	12
Trypsin plus 5 cc. of the incubated mixture of the inhibitor solution and tubercular plasma (added to substrate after 30 minutes)	21

The results show that the inhibitor is inactivated by some agent in tubercular plasma which in all probability is a polypeptidase. This was further confirmed by incubating normal plasma containing the inhibitor with tubercular plasma. The results are given in Table VII.

TABLE VII

	Activity expressed as 'Increase in N.P.N. for 100 cc. Plasma'
Tryptic activity of normal plasma	25 mg.
Activity of tubercular plasma	36
Activity of normal plasma after incubating for one hour with tubercular plasma (5 cc. normal plus 5 cc. tubercular)	74

The increase in the tryptic activity of the incubated mixture of equal quantities of normal and tubercular plasma is more than the arithmetic sum of the two activities individually, but is approximately double the activity of the tubercular plasma. This shows that the inhibitor present in normal plasma has been inactivated by tubercular plasma.

This result leads naturally to an investigation of polypeptidase activity of tubercular plasma as compared with normal plasma. The polypeptidase activity of a number of tubercular plasma was determined by a method similar to that of Grassman and Dyckerhoff (5) using dl-leucylglycylglycine as the substrate.

0.61 grams of dl-leucylglycylglycine was treated with 1.0 cc. of 0.25 N Ammonia solution and 1.0 cc. of N Ammonium chloride solution and to this was added 0.85 cc. of 0.33 N Phosphate buffer solution of *p*H 7.0 and made up to 50 cc. 5 cc. of this solution was warmed to 40°C and 5 cc. of tubercular or normal plasma were added. 4 cc. of this mixture was titrated immediately in 90% alcohol with 0.01 N KOH using thymolphthalein as indicator. After incubating the mixture for two hours another 4 cc. of this mixture was likewise titrated. The difference between the two titration values gives the polypeptidase activity of the plasma. The results are given in Table VIII.

TABLE VIII

Polypeptidase activity of tubercular and normal plasma.

	Normal Volume of 0.01N KOH	Tubercular Volume of 0.01N KOH
(1)	Nil	0.8 cc.
(2)	Nil	0.6 cc.
(3)	Nil	0.9 cc.
(4)	0.1 cc.	0.8 cc.
(5)	0.1 cc.	0.8 cc.

There is a significant polypeptidase activity in tubercular plasma while in normal plasma the enzyme is absent.

This important change in the blood of a tubercular patient can be used for diagnostic purposes. Instead of measuring the polypeptidase activity which requires the use of synthetic polypeptides, the absence of the inhibitor, which can be proved by determining the tryptic activity of plasma before and after treatment with trichloracetic acid, can be used as a diagnostic test for tuberculosis.

Similar experiments as the above were carried out with plasma from typhoid patients. The inhibitor was found to be present, the plasma was not capable of inactivating the inhibitor and there was negligible polypeptidase activity.

Summary and Conclusions

(1) Proteolytic activity of plasma from tubercular and typhoid patients have been determined by studying the increase in 'non-protein nitrogen' of the sterile plasma after incubation for 24 hours.

(2) In both the diseases a significant increase in the proteolytic activity over that of the normal plasma has been observed.

(3) This increase in the proteolytic activity in tuberculosis has been found to be due to the absence of the trypsin-inhibitor while in typhoid the increase is due to a greater amount of the enzyme circulating in the blood, because the inhibitor was found to be present.

(4) The absence of the inhibitor in tuberculosis is due to the capacity of the plasma to inactivate the inhibitor. The tubercular plasma was found to destroy the trypsin inhibitor *'in vitro'*, in both normal plasma as well as the trypsin-inhibitor isolated from beef blood.

(5) Polypeptidase was found to be present in tuberculosis plasma and not in normal or typhoid plasma. The absence of the inhibitor in tuberculosis plasma is therefore due to the presence of polypeptidase which acts upon the inhibitor known to be a polypeptide of low molecular weight.

(6) It is suggested that this finding of the absence of trypsin-inhibitor in tuberculosis plasma can be used as a diagnostic test for tuberculosis.

(7) This test can be carried out by determining the tryptic activity of the patient's plasma before and after treatment with 2.5% trichloracetic acid.

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EFFECT OF ALLOXAN ON INSULIN

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Dunn (1) *et ai* have reported that parenteral administration of Alloxan is followed by complete necrosis of the pancreatic islet tissue, hyperglycaemia and glycosuria, simulating the symptoms of 'Diabetus Mellitus'. Jacobs (2) observed that the intravenous injection of Alloxan brings about a profound fall in blood-sugar which is succeeded by hyperglycaemia.

Hughes, Ware and Young (3) have elucidated the mechanism by which Alloxan acts in the above manner. The immediate effect of the administration of Alloxan intravenously to a fasting rabbit is to raise the blood-sugar to between 150 to 250 mgs. per 100 cc. This phase is followed by a fall in blood-sugar coming to hypoglycaemic levels in about 8 hours after injection. This initial hyperglycaemia has been explained by Hughes *et al* (loc cit) to be possibly due to the release of adrenaline in the body. The hypoglycaemic action of Alloxan is considered to be due to the slow release of preformed Insulin from dying islet cells.

Alloxan is an oxidizing agent having a special affinity for sulfhydryl groups. Purr (4) has found that it causes reversible inactivation of Papain and cathepsin, the activity of which depends on the presence of-SH groups. With the nitroprusside test for-SH groups, the inactivation was attributed to the disappearance of the-SH groups.

Jensen and Evans (5), Freudenberg *et al* (6) and Du Vigneau *et al* (7) have all reported that cysteine, glutathione and thioglycollic acid will inactivate Insulin. Insulin in the native and fully active state is in the oxidised condition and no free-SH groups are detectable. When the hormone is acted upon by thioglycollic acid (or any of the above reducing agents) at *p*H near 2, the first phase is the conversion to the reduced state if precautions are taken not to denature the protein. Later in the process, the protein suffers marked physical changes e.g. decrease in solubility and increase in viscosity and gel formation which can be regarded as criteria for the commencement of denaturation. The point at which the hormone can be brought to the reduced condition without denaturation has been worked out by Stern and White (8). This stage is reached after about 150 minutes reduction by thioglycollic acid. The protein at this stage contains about 2 to 3 cysteine equivalents per Insulin molecule and about 50% of the original activity is retained ; complete inactivation would then require more drastic changes in the molecule, possibly leading to complete denaturation. Compared with the original S-S insulin,

the reduced product, just before the commencement of denaturation, has practically identical content of tyrosine, free amino groups and total sulphur. The molecular size, the iso-electric point, the viscosity and the ultraviolet absorption spectrum are also found to be unchanged. These findings support the conclusion that the only alteration produced by the reaction of insulin with thioglycollic acid is a reduction of a small number of dithio linkages to sulfhydryl groups.

In view of the special affinity of Alloxan for-SH groups, the possibility of reactivating Insulin, partially inactivated by thioglycollic acid, up to the stage at which denaturation just commences, is worth investigation. The results of such a study may throw light on the mechanism of action of Alloxan.

EXPERIMENTAL

10 mg. of amorphous Insulin were dissolved in acid saline and the *p*H adjusted to about 2. Thioglycollic acid was added to this and the reaction allowed to proceed for about two hours in the absence of air. The excess of thioglycollic acid was removed by acetone as described by Stern and White (loc cit). The solubility and viscosity of the resulting product was found to have been altered very little, thereby assuring that the denaturation was reduced to a minimum.. The activity of this product was determined by the rabbit blood-sugar method and found to be about 9 units per mg. 2 mg. of this partially inactivated, but not denatured Insulin was dissolved in 10 cc. water and added to 5 cc. of a solution of Alloxan containing 50 mg. The *p*H of this solution was adjusted to 7 and incubated for 1 hour at 30°C. The insulin potency of this solution was accurately tested by the rabbit blood-sugar method. The results are given in Table I.

TABLE I

	Potency of Insulin.
(1) Amorphous Insulin before treatment with thioglycollic acid.	18 units per mg.
(2) Insulin, after treatment with thioglycollic acid for 2 hours and the excess of the reagent removed by Acetone treatment.	9 units per mg.
(3) 2 mg. of (2) in 10 cc. plus 5 cc. of Alloxan (50 mg.) incubated for 1 hour at 30°C *p*H 7.	2 units per cc. or 15 units per mg. of the insulin; (the total volume of the solution being 15 cc. and the solid insulin (2) added being 2 mg.)

The thioglycollic acid treated Insulin appears to be activated by Alloxan. The activity of the product has been increased from 9 units per mg. to 15 units, the original activity being 18 units. This reactivation brought about by Alloxan should be ascribed to its capacity to specifically oxidise SH groups to SS groups. In determining the potency of solution (3) in Table I, the whole solution containing Alloxan was injected to the rabbit. There is every possibility of Alloxan interfering with the blood-sugar reducing capacity of Insulin which is the measure for determining the potency. It is therefore essential to see whether Alloxan in the dosage employed has any effect on the blood sugar of the rabbit or interferes in

any way with the quantitative effect of Insulin alone. The concentration of Alloxan in solution (3) is 3.3 mg./cc. 0.5 cc. of this solution was injected subcutaneously to the rabbits for the determination of the potency of the solution. Hence the absolute amount of Alloxan injected was 1.65 mg. The effect of double this amount of Alloxan by itself or with one unit of Insulin on the blood-sugar of the rabbit has been determined. The results are given in Table II.

TABLE II

Effect of Alloxan, and Alloxan and Insulin on the blood-sugar of rabbits

	Average % of Blood-sugar reduction.
Insulin 1 unit.	34%
Insulin 1 unit plus 5 mg. Alloxan	31.5%

Alloxan in dose of even 5 mg. had no effect on the blood-sugar. It is therefore evident that Alloxan has the property of reactivating Insulin which has been partially inactivated by reduction with thioglycollic acid under controlled conditions ensuring that no appreciable denaturation takes place.

If the treatment with thioglycollic acid is continued for a period of three hours, the resulting product shows increased viscosity and markedly decreased solubility, the two conventional criteria for denaturation. The activity of this product is 7 units per mg., a value which is not very much less than the product obtained after 150 mins. treatment. Now that the denaturation has taken place, although there is not much increased reduction of SS groups, it will be interesting to determine whether Alloxan has the property of reactivating this product. The results are given in Table III.

TABLE III

	Potency.
(1) Insulin (original)	18 units per mg.
(2) Insulin after treatment with thioglycollic acid for three and half hours.	7 units per mg.
(3) 2 mg. of (2) in 10 cc. saline plus 50 mg. Alloxan in 5 cc., incubated for 1 hour at *p*H 7.	1 unit per cc. or about 7 units per mg. of preparation (2).

There is practically no reactivation of (2) after treatment with Alloxan. The inactivation due to denaturation as well as reduction of S-S groups cannot be reversed by Alloxan while the partially reduced but not denatured Insulin can be reactivated by Alloxan. Having established that Alloxan reactivates partially reduced Insulin, the application of this important fact in explaining the already discovered action of Alloxan on the blood-sugar of rabbits is next considered. Among the various modes of destruction of Insulin, the part played by glutathione and cysteine does not seem to have received the required attention.. In the reduced state, glutathione can inactivate Insulin. The reactivation of Insulin, inactivated by such a reduction by Alloxan has been demonstrated *'in vitro'*. It is quite likely that such a reactivation takes place *'in vivo'* after the administration of Alloxan.

As this reaction takes one hour or more, the hypoglycaemic effect of Alloxan can be observed only after a few hours, a fact observed in the blood-sugar of rabbits injected with Alloxan.

The immediate hyperglycaemia after Alloxan administration has been explained by Young *et al* to be possibly due to the liberation of Adrenaline. The subsequent hypoglycaemic effect is considered to be due to the slow release of preformed Insulin from dying islet cells. While this explanation supported by experimental data is the most probable one, the possibility that Alloxan might reactivate the partially inactivated Insulin (by glutathione normally present in blood) exists.

In order to test this possibility, the action of lower doses of Alloxan (not such a low dose as used in Table II) which might not effect the islet cells, has been investigated. The dosage of Alloxan employed by Dunn *et al* in order to produce the classical symptoms of diabetus mellitus and at which the islet cells are damaged is 200 mg. per kilo. A dose of 50 mg. per kilo has been employed in our experiments and the results are given in Table IV.

TABLE IV

Dose of Alloxan.		Blood-sugar in mg. per 100 cc. at intervals of				
		0 hr.	½ hr.	1 hr.	2 hrs.	3 hrs.
(1)	50 mg. per kg.	100	106	92	89	87
(2)	—do—	94	100	86	81	85
(3)	—do—	125	129	112	105	104
(4)	—do—	118	125	105	100	102
(5)	—do—	108	112	102	93	95
(6)	—do—	98	103	91	86	83
Average percentage reduction		...	...	9%	13.5%	13%

In every case there is a gradual fall in blood-sugar after the intravenous administration of 50 mg. of Alloxan per kg. weight of the rabbit. In the first half hour a very slight rise is observed which may not have any significance, as such small changes might be observed in rabbits without any treatment. The dose of 50 mg. per kilo is not known to produce any damage of the islet cells and the reduction of the blood-sugar should therefore be attributed to some action of Alloxan other than its capacity to release preformed Insulin from dying islet cells. This hypoglycaemic effect of small doses of Alloxan can be explained in two ways:

1. Increased production of Insulin in the pancreas by a possible mitotic action of Alloxan as visualised by Hughes *et al* (loc cit) or,
2. Activation of inactive Insulin present in the blood.

In the absence of evidence for the specific mitotic action of Alloxan in the islet cells, the second possibility is worthy of consideration. There is always active and Inactive Insulin present in the circulating blood. If by some method, the inactive Insulin can be activated, a reduction in blood-sugar is to be observed. As such a result has been demonstrated by the intravenous administration of Alloxan (50 mg. per kilo) it can be stated that the substance has this activating property, particularly if the inactivation has been brought about by reduced glutathione or cysteine.

In support of this hypothesis the following experiment was carried out. The hypoglycaemic effect of 0.5 unit per kg. of Insulin was given subcutaneously, and 50 mg./kg. of Alloxan was given intravenously. The blood-sugars of these rabbits were followed for a period of 3 hrs. The results are given in Table V.

TABLE V

	Average Blood-sugar in mg. per 100 cc. after various intervals.				
	0 hr.	1 hr.	2 hrs.	3 hrs.	4 hrs.
0.5 unit per kg. Insulin.	122	96	85	90	104
0.5 unit per kg Insulin subcutaneously and 50 mg. per kg of Alloxan intravenously.	118	99	62	79	94

TABLE VI

(*Computed from the results of Table V*)

	Average percentage of Blood-sugar reduction at the end of 2 hrs.
0.5 unit per kg Insulin subcutaneously.	30%
0.5 unit per kg Insulin subcutaneously and 50 mg. per kg of Alloxan intravenously.	47.5%

The hypoglycaemic effect of Insulin and Alloxan at the end of 2 hrs. is greater than that of a same dose of Insulin alone. When the same dose of Alloxan is administered alone, the percentage of blood-sugar reduction is 13.5, the effect of Insulin alone is 30%, the sum total of these two is 43.5%, while the observed effect is 47.5%. If this increase of 4% over the arithmetic sum of the effects of Insulin and Alloxan separately is regarded as significant, it can be attributed to the possible activating influence of Alloxan on the extraneous injected Insulin which might have been partially inactivated by thiol reductions, in addition to the activation of Insulin circulating in the blood in the inactive condition.

The experiments reported provide indirect evidence for the probable role of mild doses of Alloxan as an activator for Insulin, assuming that there is always some amount of reversibly inactivated Insulin circulating in the blood. If there was an accurate method of estimating Insulin in blood, direct evidence for the activating influence of Alloxan could be produced by incubating Alloxan with blood and determining the increase if any, in the blood-sugar reducing capacity of the blood.

Summary and Conclusions

(1) The *'in vitro'* action of Alloxan on Insulin, partially inactivated by the action of thioglycollic acid under controlled conditions without appreciably denaturing the protein, has been studied.

(2) When the reduction is allowed to proceed for 2 hrs. the activity of the Insulin is decreased from 18 units per mg. to 9 units per mg. Alloxan activates this Insulin and the potency is increased to 15 units per mg. In view of the fact that the inactivation of Insulin by thioglycollic acid is due to the reduction of S-S groups, the reactivation by Alloxan is ascribed to the oxidation of S-H groups to S-S groups in which state alone the native Insulin protein is active.

(3) If the reduction of Insulin by thioglycollic acid is carried out more drastically over a longer period resulting in appreciable denaturation of the protein, Alloxan has no activating effect although the inactivation of Insulin is not very much more than in the previous case.

(4) The intermediate hypoglycaemic effect of Alloxan observed in between the initial and the final hyperglycaemic effects has been explained by Hughes *et al* (loc cit), to be due to the slow release of preformed Insulin from dying islet cells. In addition to this most plausible reasoning, the possibility of this action of Alloxan being due to its activating influence on the reversibly inactivated Insulin present in the circulating blood, has been considered.

(5) Indirect evidence for such a possibility has been attempted.

(6) Intravenous injection of Alloxan (50 mg./kg.), in doses far below the dose (200 mg./kg.) known to produce extensive damage of the islet cells, produces a noticeable reduction in the blood-sugar of rabbits.

(7) The blood-sugar reducing capacity of 0.5 units per kg. of Insulin is also increased if it is immediately followed by an intravenous injection of Alloxan (50 mg. per kg.).

(8) These results are suggestive of the possibility of Alloxan in lower doses acting as an activator for any reversibly inactivated Insulin present in blood.

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COMPARATIVE ACTION OF TRYPSIN ON AMORPHOUS INSULIN, CRYSTALLINE-ZN-INSULIN, PROTAMINE-ZN-INSULIN AND GLOBIN INSULIN

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The prolongation of the hypoglycaemic effect of Insulin when administered in various forms of the hormone is in the order of:

(1). Protamine-zn-Insulin,

(2). Crystalline-zn-Insulin,

(3). Amorphous Insulin. (Scott, 1).

Globin-Insulin, a product recently introduced by Burrough's Wellcome has been found to have the prolongation effect less than that of protamine-zn-Insulin and more than that of crystalline-zn-Insulin. Beyond the statement that this might possibly be due to the insolubility of the complex protamine-zn-Insulin the reasons for this graded prolongation effect of the various forms of Insulin have not been satisfactorily explained. The possibility that the different rates of destruction of Insulin by plasma-trypsin when administered in the various forms may account for the variations in the duration of Insulin effect has not been considered. As the author was engaged in a systematic study of the proteolytic system in blood, it occurred to him to test this hypothesis. This has been done in the present paper by studying the rate of destruction of Insulin as well as the increase in non-protein nitrogen when the various Insulin preparations were subjected to the action of trypsin.

MATERIALS AND METHODS

The following solutions were used:

(1). Protamine-zn-Insulin, manufactured by Lillys, having a potency of 40 units per cc.

(2). Crystalline-zn-Insulin solution having a potency of 40 units per cc. was prepared from the International Insulin Standard.

(3). Amorphous Insulin solution of a potency of 40 units per cc. was prepared from Amorphous Insulin powder having a potency of 15 units per mg.

(4). Globin-Insulin solution manufactured by Burrough's Wellcome and Company, having a potency of 40 units per cc.

(5). B.D.H. Trypsin, 0.5% solution. (A weak trypsin solution was used as the experiments were designed to test the comparative action, when subjected to very mild tryptic digestion similar to that possibly taking place in plasma).

The rate of destruction of Insulin by trypsin was studied by the rabbit blood-sugar method. The percentage of blood-sugar reduction after administration of each test solution was determined in 15 rabbits. The rate of proteolytic action was determined by the increase in non-protein nitrogen. In the case of Globin-Insulin, however, only the rate of Insulin inactivation was determined and not the increase in non-protein nitrogen, because the globin part of the solution will also give rise to non-protein nitrogen as a result of tryptic action. 1 cc. of trypsin solution was added to 5 cc. of each of the above four solutions. Non-protein nitrogen was determined in each case immediately after adding trypsin solution. The solutions were all incubated at 37°C for half an hour. The *p*H of all the solutions was 7.2. The hypoglycaemic effect of each of these solutions as well as the N.P.N. were determined after incubation. In the case of Protamine-zn-Insulin, the hypoglycaemic effect was determined after acidification and dilution in order to break the suspension. This will give a measure of the potency of Insulin. Otherwise if directly used, the hypoglycaemic effect will not be a true measure of the potency. The average percentage of blood-sugar reduction will give a measure of the potency retained in each solution after tryptic digestion. The lower the effect, the greater the inactivation. The results are given in Table I.

TABLE I

Each solution is incubated for 30 mins.

	Average percentage of blood-sugar reduction (2 cc. of each mixture is diluted to 16 cc.) 0.5 cc. of this solution is injected into rabbits.	Increase in N-P-N calculated for 100 mg. of Insulin.
(1) Protamine-zn-Insulin plus trypsin	31.4	2.5 mg.
(2) Crystalline-zn-Insulin solution plus trypsin	24.9	3.4
(3) Amorphous Insulin plus trypsin solution	19.2	4.5
(4) Globin Insulin plus trypsin solution	28.2	...

The rates of inactivation of Insulin as well as the digestion of the Insulin protein by a dilute solution of trypsin are in the order of Amorphous Insulin, Crystalline-zn-Insulin and Protamine-zn-Insulin. The Globin-Insulin comes in between Crystalline-zn-Insulin and Protamine-zn-Insulin, in the rate of inactivation. The rate of digestion of the Insulin protein in globin-Insulin could not be carried out as the protein globin will also be digested and it is not possible to apportion the digestion of Insulin protein from the total digestion of both the proteins. The susceptibility of Insulin to tryptic attack is different in each of the above preparations. This result is very interesting in view of the fact that it runs parallel with the recorded findings, on the duration of the hypoglycaemic effect of each preparation.

The close parallelism between the two findings affords strong evidence in support of the hypothesis that the duration of the effect of Insulin, if not also the intensity, depends upon the rate of destruction by trypsin circulating in the blood.

The reason why Insulin in different forms or in combination with other substances should behave differently in its liability to tryptic action, is not far to seek.

In the case of Protamine-zn-Insulin, it was thought that its prolongation effect was due to the insolubility of the complex. The possibility that Protamine might act as a trypsin inhibitor has to be considered.

In the case of Globin-Insulin, which is a clear solution, the protection offered to the hormone can be explained on the basis of the author's work, wherein he has demonstrated that other proteins which are more susceptible to tryptic action might be able to protect Insulin which is comparatively a more resistant protein.

The effect of Protamine on the tryptic digestion of casein at a *p*H of 7.2 was investigated in order to ascertain whether Protamine acts as a trypsin inhibitor. A solution of protamine containing 2 mg. per cc. was prepared. The substrate used was 2% casein solution. A 5% B.D.H. trypsin solution was the enzyme. The results are given in Table II.

TABLE II

		Increase in N.P.N. in the digest.
Incubated for 2 hours at 37°C.	10 cc. Casein solution plus 2 cc. trypsin solution plus 2 cc. Buffer of *p*H 7.2 plus 1 cc. water.	9.2 mg.
Incubated for 2 hours at 37°C.	10 cc. Casein plus 2 cc. trypsin solution plus 2 cc. Buffer plus 1 cc. Protamine solution.	14.8

It is evident from the results that protamine is a trypsin inhibitor at *p*H 7.2. In the experiments reported, the protamine has been directly added to the enzyme substrate mixture and yet the inhibitory action is observed unlike the other trypsin inhibitors; (1) Polypeptide inhibitor from blood and (2) Heparin, both of which

have to remain in contact with the enzyme for a period of 30 minutes, before the addition of the substrate, in order to exhibit their inhibitory actions. The important difference between protamine on the one side and the other two trypsin inhibitors on the other side, accounts for the inability of the latter two inhibitors to exert any significant prolongation effect (Iyengar 2.) when mixed with Insulin and injected into rabbits. These two inhibitors have no opportunity to combine with plasma trypsin before Insulin is administered in order to be able to exert their inhibitory action.

SUMMARY

(1). The comparative action of Trypsin on amorphous Insulin, Crystalline-zn-Insulin, Protamine-zn-Insulin and Globin-Insulin has been studied by following both the inactivation of Insulin as well as the increase in non-protein-nitrogen, the latter result being expressed as so many mg. for 100 mg. of Insulin.

(2). The rates of inactivation of Insulin as well as the digestion of Insulin protein are in the order of (*i*) Amorphous Insulin, (*ii*) Crystalline-zn-Insulin (*iii*) Globin-Insulin and (*iv*) Protamine-zn-Insulin.

(3). Protamine has been found to be an inhibitor of the tryptic digestion of casein at *pH* 7.2.

(4). The inhibitory effect of protamine is noticed even when it is directly added to the enzyme-substrate mixture unlike the other two inhibitors (*a*) Polypeptide from blood and (*b*) Heparin both of which have to remain in contact with enzyme for a period of 30 minutes before the substrate is added.

(5). This important difference between protamine on the one side and the other two trypsin-inhibitors on the other side accounts for the inability of the latter two substances to exert any significant prolongation effect when mixed with Insulin and injected into rabbits.

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OBSERVATIONS ON ASCORBIC ACID. PART IV. THE EFFECT OF CERTAIN FACTORS ON ASCORBIC ACID PRODUCTION DURING GERMINATION OF SEEDS

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It is now well recognised that germinating seeds are strongly antiscorbutic as compared to dormant seeds (1-8). It has been observed that dicotyledonous seeds synthesise ascorbic acid to a much greater extent than monocotyledonous seeds. Much of this work has been carried out on peas, pulses and other leguminous seeds. Muthanna and Ahmad (9) showed that even common cereals can become good sources of ascorbic acid on germination, the rate of increase during germination being of the same order as that observed in leguminosae.

The production of ascorbic acid is dependent to some extent upon the nature of the seed and the conditions of germination. It has been shown that Mung (green gram) gives greater synthesis of ascorbic acid as compared to other cereals under identical conditions. The effect of environmental factors such as light, *p*H, temperature has been studied by a number of investigators (10-13). It has been observed that light has an appreciable effect upon the synthesis of ascorbic acid while lower temperatures which hinder rapid germination and growth tend to increase the vitamin C content of the seeds.

The effect of traces of mineral salts in the nutrient media has also been investigated (14). Manganese appears to form an important link in the biosynthesis of ascorbic acid from its precursors.

In all these investigations the effect of environmental factors has been studied using whole seeds for germination. In their classical work, Brown and Harris (15) showed that the embryos can be excised out of the endosperm and cultivated on nutrient solutions. This technique has been used in investigations of the precursors of ascorbic acid in metabolising tissues (16). In this investigation we have largely used this technique for the study of the effect of different environmental factors upon the synthesis of ascorbic acid during germination.

Effect of light.—In this experiment whole seeds were used and were allowed to germinate in (a) total darkness, (b) shade and (c) in direct sunlight during the day. Samples of seeds were removed at different intervals and their vitamin C content estimated by the usual technique. The results are shown in Table I.

TABLE I

Action of light in biosynthesis of ascorbic acid during germination.

Name of cereal	Time of germination. (days)	Mg. ascorbic acid per 100 g. of seed germinated in		
		Total darkness.	Shade.	Direct sunlight.
Bengal gram				
	2	...	46.0	...
	3	56.2	69.1	118.2
	4	83.3	89.8	128.7
	6	110.2	132.6	186.0
	8	70.4	120.1	230.1
Mung (Green gram)				
	2	...	44.0	...
	3	163.7	152.9	214.2
	4	165.0	206.2	257.1
	5	190.1	206.8	326.0
	9	161.3	182.5	330.0
Soya bean				
	3	80.0	71.1	...
	5	71.8	91.1	70.4
	8	66.0	82.4	82.1
	9	52.2	60.8	44.4

It would be of interest to note that both Bengal gram and Mung showed a striking increase in ascorbic acid synthesis in the presence of sunlight, while soya bean showed practically no difference in the quantity of vitamin C synthesised in the presence or absence of light. In the first two seeds the quantity of ascorbic acid began to develop markedly after the second day. The amount of ascorbic acid in darkness and in shade increased up to the fifth day, which marked the period of maximum synthesis, after which it began to diminish. After the third day the primary leaves were developing noticeably. These became green in sunlight, while in the shade they were greenish yellow and in darkness quite yellow. In sunlight ascorbic acid synthesis continued up to the eighth day, the end of the experiment. At this period the amount of ascorbic acid synthesised in sunlight was nearly double the maximum value obtained in darkness. Soya bean, on the other hand, did not show any marked effect of light. The maximum value was obtained after 3—5 days. The soya bean seeds used contained 43.2 per cent protein and 19.5 per cent fat as against 17.1 per cent protein and 5.3 per cent fat for Bengal gram, and 24.0 per cent protein and 1.3 per cent fat in Mung. The carbohydrate content of soya bean is thus low and it is possible that the embryo has to depend largely upon protein and fat, the assimilation of which is slow. The effect of sunlight which is very marked in seeds containing readily assimilable carbohydrate, is almost negilgible in this case.

Effect of traces of mineral salts.—The stimulating effect on the biosynthesis of ascorbic acid reported by earlier workers was based on experiments using germinating whole seeds. As mentioned above in these experiments the embryos were excised out of the seed and planted on semi-solid sterile medium made with agar agar and Knopp's solution, and two different carbohydrates glucose and mannose in 5 per cent concentration. The effect of different concentrations of mineral ions was investigated in the above. The technique requires great care for the maintenance of aseptic conditions in the cultures. Fungus and bacterial infections occur with great avidity and the results are vitiated in contaminated cultures. The results are summarized in Table II.

TABLE II

Effect of various mineral ions on the biosynthesis of ascorbic acid in green gram embryo cultures.

Mineral ion used and concentration.	Mg. ascorbic acid per gram of wet seedling tissues after 7 days' growth.			
	Medium with glucose.		Medium with mannose.	
	Without mineral ion.	With mineral ion.	Without mineral ion.	With mineral ion.
Cobalt chloride				
.05 per cent	2.30	1.60	4.57	2.72
.02 ,,	3.14	1.71	3.48	2.27
.01 ,,	1.28	1.24	2.05	1.40
.005 ,,	0.75	0.65	0.81	0.92
Boric acid				
.05 ,,	0.75	0.61	0.86	0.74
.02 ,,	0.70	0.58	0.85	0.75
.01 ,,	1.33	1.61	1.90	2.57
Manganese chloride				
.05 ,,	3.63	2.02	3.09	2.69
.02 ,,	0.54	0.58	0.55	0.62
.01 ,,	1.22	1.47	1.50	1.78
Calcium fluoride				
.02 ,,	0.50	0.50	0.55	0.56
.01 ,,	0.62	0.62	0.63	0.63
.005 ,,	0.52	0.51	0.61	0.60

Mung was selected for these experiements because in this seed the largest synthesis of ascorbic acid has been found to occur during germination. The figures shown in Table II are averages of two experiments conducted side by side. It is found that larger concentrations of mineral ions actually lowered ascorbic acid production. Stimulating effect was noticeable with boric acid in low concentrations of 0.01 per cent and of manganese chloride in concentrations of .02 and .01 per cent. Calcium fluoride appeared to have no effect at all while cobalt chloride was found to be toxic to the development of chlorophyll in the absence of which ascorbic acid synthesis is retarded as was also shown by experiments summarized

in Table I. The green parts of plants are generally known to be richer in ascorbic acid than non-green parts (17-18).

Effect of chlorophyll, carotenoids and vitamins.—In order to investigate further the effect of chlorophyll upon the synthesis of ascorbic acid in growing plant embryos, a sample of pure chlorophyll-a was prepared according to the method of Willstätter and dispersed in the medium used for the cultures. Similarly in another set of culture media β-carotene was mixed in a fine state of dispersion. Pure thiamin chloride was used in another series of experiments. The results are shown in Table III.

TABLE III

Effect of chlorophyll, β-carotene and vitamin B_1 on the biosynthesis of ascorbic acid in green gram embryo cultures.

Substance and concentration.	Mg. ascorbic acid per gram of wet seedling tissues after 7 days' growth.			
	Medium with glucose		Medium with mannose.	
	Without substance.	With substance.	Without substance.	With substance.
Chlorophyll-a				
.01 per cent	0.63	0.72	0.64	0.85
.003 ,,	0.57	0.59	0.75	0.75
β-Carotene				
.004 ,,	0.31	0.31	0.33	0.33
.002 ,,	0.31	0.31	0.33	0.33
Vitamin B_1				
.004 ,,	1.19	1.20	1.54	1.52

The figures in the table are averages of the results of two consecutive experiments conducted side by side. In the concentrations used and under the conditions of the experiments β-carotene and vitamin B_1 seemed to have no effect on ascorbic acid production. Chlorophyll-a in a concentration of 0.01 per cent showed significantly stimulating effect on the biosynthesis of ascorbic acid.

Effect of some sugars.—This subject is of particular interest because it is calculated to throw light on the nature of percursors of ascorbic acid in biosynthesis in nature. Ray (16) was the first to use this technique in ascertaining the nature of percursors involved in the process of germination. He found that hexoses, particularly, mannose, lead to a moderately high production of ascorbic acid in germinating embryos and serve as a precursor of vitamin C in their tissues. The following experiments are essentially a repetition of Ray's work. In this connection reference may be made to the studies of Johnshin and Potter (19) who investigated galacturonic acid as a possible precursor for vitamin C. For making the culture media we used agar agar with Knopp's solution and different sugars in 5 per cent concentration. The results are shown in Table IV.

TABLE IV

Effect of different sugars on the biosynthesis of ascorbic acid in green gram embryo cultures.

Sugar.	Degree of growth.	Mg. ascorbic acid per g. wet seedling tissues after 7 days' growth.	Per cent increased synthesis.
Control without any sugar	++	0.61	...
Maltose	+++	1.24	103.6
Mannose	++	1.20	96.6
Glucose	++	3.00	391.8
Xylose	+	0.70	14.8
Lactose	++	1.05	72.7

The figures are averages of the results of two and sometimes three consecutive experiments. Increased synthesis of ascorbic acid occurred in practically all the cases. The highest figure was obtained in the case of glucose, the next being maltose and mannose, while relatively smaller increase was found in the case of lactose and xylose.

These experiments cannot be stated to show a definite relationship between the degrees of growth of the embryos and the synthesis of ascorbic acid. Maltose which induced the highest degree of growth was not found to be as good as glucose in ascorbic acid production. On the other hand in the case of xylose both growth and the synthesis of ascorbic acid were the lowest.

CONCLUSIONS

The effect of a number of factors involved in the biosynthesis of ascorbic acid in germinating seedlings or embryos of plant seeds has been studied. It is found that when the seeds are cultivated in light and more so in direct sunlight considerable increase in the synthesis of ascorbic acid in their tissues occurs. Bengal gram and green gram seeds showed over 100 per cent more ascorbic acid when cultivated in sunlight, than when grown in darkness. While in darkness or in shade the maximum ascorbic acid content was reached in 5 to 6 days, the cultures exposed to direct sun during the day continued to increase their vitamin C content for as long as 8 to 9 days, the experiments having been discontinued after the ninth day. It was striking to note that soya bean seeds did not show any increase in ascorbic acid synthesis as a result of exposure to light. This behaviour may be related to its high protein and fat content and low content of readily assimilable carbohydrate. As can be expected there was greater production of chlorophyll in germinating seeds exposed to direct sunlight.

The effect of a number of mineral salts in very small concentrations of biosynthesis of ascorbic acid in plant embryos growing on artificial semi-solid media containing agar agar Knopp's solution and 5 per cent glucose or mannose, were also investigated. Larger concentrations than 0.02 per cent seemed to retard the

synthesis, while in lower concentrations of boric acid or manganese chloride an increased synthesis was found. Cobalt chloride, however, even in as small concentrations as 0.005 per cent retarded both chlorophyll and ascorbic acid synthesis. Calcium fluoride appeared to be completely inert in this respect. The effect of the addition of chlorophyll, β-carotene and thiamin chloride to the culture media was also investigated. Of these only chlorophyll in a concentration of 0.01 per cent seemed to have a somewhat augmenting effect.

The addition of a number of sugars, both mono and disaccharides to the culture media was studied. While relatively small amounts of ascrobic acid are synthesised by the embryos in the absence of sugars, all the sugars used increased the synthesis of ascorbic acid in embryo tissues. The high figures were obtained in the case of glucose, maltose and mannose, comparatively lower for xylose and lactose. In the case of xylose which showed the smallest increase in ascorbic acid synthesis the growth of the embryo was also the poorest.

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A MODIFIED PROCEDURE FOR THE ESTIMATION OF NICOTINIC ACID IN BIOLOGICAL MATERIALS

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A colorimetric method for the estimation of nicotinic acid in biological materials involving the use of cyanogen bromide and aniline was described by Swaminathan (1). The method is based on the observation of Kœnig (2) that a coloured compound is formed when the pyridine nucleus reacts with cyanogen bromide and a primary or secondary aromatic amine. König pointed out that the reaction in any form required the use of cyanogen bromide but in place of aniline any other aromatic amine could be used. A number of other workers have proposed modifications of the cyanogen bromide method based on (i) the use of other aromatic amines in place of aniline or (ii) involving the use of different procedures for the preparation of extracts of biological materials for the colorimetric estimation. β-Naphthylamine, metol and *p*-aminoacetophenone have been employed, in place of aniline, by Euler, Schlenk, Heiwinkel and Högberg (3), Bandier and Hald (4), and Harris and Raymond (5) respectively. Bandier and Hald (4), chose metol, as according to them it gave a stable colour. Harris and Raymond (5) pointed out that the intensity of colour obtained with *p*-aminoacetophenone was greater than that obtained with aniline or metol and hence *p*-aminoacetophenone was preferable to other amines for the reaction. In later publications Swaminathan (6, 7) made a critical study of the colour development with aniline, β-naphthylamine, *p*-aminoacetophenone and metol, and showed that the colour reaction could be carried out under four different conditions: (i) in aqueous neutral medium at *p*H 7, (ii) in aqueous acid medium, (iii) in neutral alcoholic medium and (iv) in acid alcoholic medium. It was pointed out that the intensity and stability of colour produced with any aromatic amine depended upon the conditions under which the colour was produced. In the present investigation, we undertook a detailed investigation of the cyanogen bromide method using still different aromatic amines in order to find out the amine giving the colour of maximum intensity and stability. The following amines were tested:

o-Aminobenzoic acid
m-Aminobenzoic acid
p-Aminobenzoic acid
Aniline
Aniline hydrochloride
p-Amino acetophenone
β-Naphthylamine
p-Toluidine
m-Toluidine
p-Chloroaniline
Naphthol amino sulphonic acid (1:4)
Naphthol amino sulphonic acid (2:6)
Methylaniline
Hexamine
Aminosuccinic acid.

Only the primary aromatic amines were found to give a colour reaction with nicotinic acid and cyanogen bromide. Of the amines tested the best results were obtained with 5 per cent aniline hydrochloride solution. The colour remained stable for 40 minutes in light and for over 3 hours in the dark. The colour was more intense than that obtained with metol or aqueous aniline and was approximately of the same intensity as that obtained with *p*-aminoacetophenone. These results confirm the findings of Swaminathan (6. 7).

EXPERIMENTAL

Preliminary experiments.—In a detailed quantitative investigation of the reaction undertaken with the object of elaborating a reliable analytical procedure, attention had to be paid to all the following several factors: (i) Proportionality of colour (verification of Beers's Law)((ii) relation of *p*H value of nicotinic acid solution to the intensity of colour produced, (iii) stability of the colour, (iv) effect of presence of inorganic salts and organic compounds to the intensity of colour, (v) effect of temperature, (vi) effect of variations in the quantity of reagents used on the colour reaction.

Proportionality of colour (verification of Beer's Law).—Varying amounts of the standard solution were taken. The colour was produced according to the procedure described later using 1.0 cc. of cyanogen bromide and 1.0 cc. of the amine reagent. The colours obtained were compared and found to be proportional for concentrations of 10 to 100 micrograms of nicotinic acid. Beer's Law was thus valid for these concentrations.

Relation between pH value of the nicotinic acid solution and intensity of the colour produced.—One cc. of a standard solution of nicotinic acid was taken in each of several 100 cc. measuring flasks. To these flasks were added different buffers having a range of *p*H values between 1.2 and 7.8 and the volume in each case was made up to 100 cc. 1 cc. of this solution at different *p*H values containing 100 micrograms was used for the colour reaction by following the procedure described later. The results are shown in Fig. 1. It is evident that the optimum *p*H range of the initial solution for the colour reaction lies between 4.5 and 6.0.

Stability of colour.—One cc. of the standard nicotinic acid containing 100 micrograms of nicotinic acid at *p*H 5-6 was taken and colour was developed by using 1 cc. cyanogen bromide and 1 cc. of amine reagent. The time taken for the colour to attain its maximum intensity was noted and the intensity of the colour was determined at frequent intervals, (the solution being kept in diffused light in the laboratory). Another experiment was done in a similar way but keeping the sample covered with a black cloth. The results are shown in Figs. 2 and 3.

FIG. 1

RELATION OF pH VALUE AND INTENSITY OF COLOUR PRODUCED

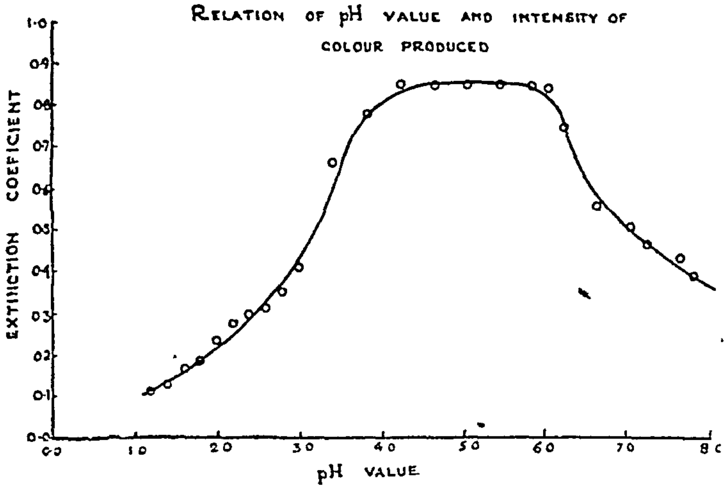


FIG. 2

STABILITY OF COLOUR IN LIGHT

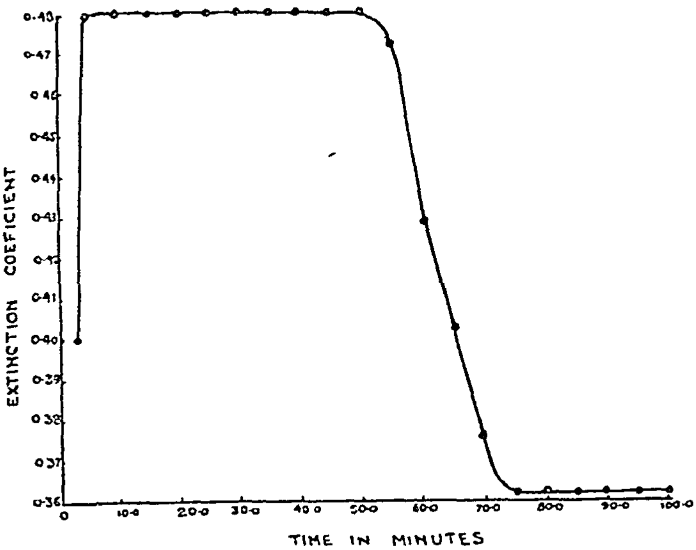
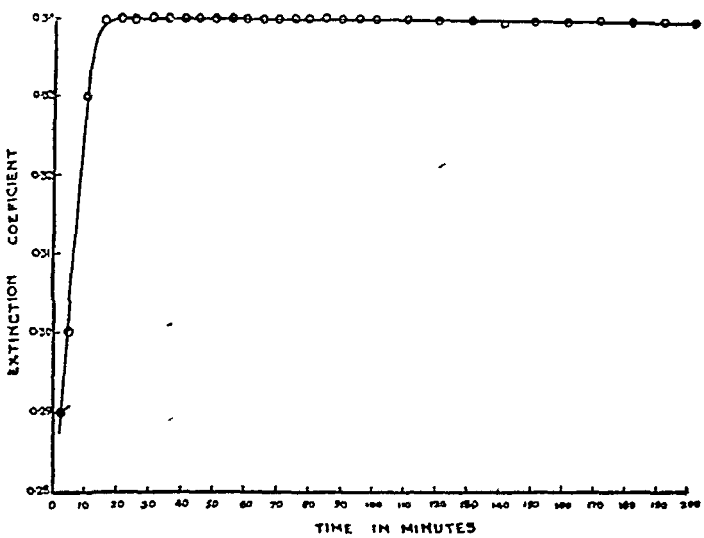


FIG. 3

STABILTY OF COLOUR IN DARKNESS



It is evident from the above results that the colour attains its maximum intensity in about 5 minutes and remains stable for over 30 minutes in diffused light and for over 3 hours in the dark.

Effect of presence of inorganic salts and organic substances on colour intensity.—Some thirty different salts were tested. These included calcium chloride, magnesium chloride, barium acetate, potassium chloride, sodium chloride, potassium sulphate, zinc sulphate, potassium dihydrogen phosphate, zinc chloride, sodium orthophosphate, sodium acetate, sodium bicarbonate, potassium iodide, potassium orthophosphate, sodium bromide, sodium hydrogen citrate, potassium sulphocyanide, manganese chloride, sodium fluoride, hydrogen peroxide, sodium carbonate, calcium lactate, sodium nitrate, ammonium chloride, potassium oxalate, lead nitrate, mercuric chloride, sodium bisulphite, sodium metabisulphite. Some of these were introduced during the preparation of extracts for analysis. It was found that the ordinary inorganic salts likely to be present in the test solutions do not interfere with the colour reaction. Salts like sodium nitrite, potassium sulphocyanide and other reducing agents were found to interfere in the development of colour, while iodides liberated iodine.

Similar experiments were performed by adding 1 cc. of 10 per cent solution of a number of organic compounds, e.g., sucrose, glucose, glycerine, urea, phenol, and formaline to the nicotinic acid solution before colour development. Of these only formaline had an adverse effect on the colour development.

Effect of temperature.—The colour was found to be rapidly destroyed by the rise of temperature to the boiling point. It was more stable below 15° and slightly less so at 30°.

Effect of variations in the quantity of reagents used.—The optimum quantity of cyanogen bromide solution and the amine reagent was found to be 1 cc. each. Increasing the quantity of the reagents did not affect the colour markedly.

Details of the method recommended.

Reagents required.

Standard Stock Nicotinic acid solution.

1 cc. solution containing 1 mg. nicotinic acid. This should be preserved in a refrigerator.

Standard Nicotinic acid solution.

1 cc. solution containing 100 micrograms of nicotinic acid. This was prepared fresh daily by diluting 1 cc. of stock standard solution to 10 cc. with water.

Aniline Hydrochloride solution 5 per cent.

5 cc. of pure aniline were taken in a conical flask and 15 cc. of concentrated hydrochloric acid (32 per cent) were added to it drop by drop, the flask being cooled in ice. Aniline hydrochloride separated. This was dissolved in water and was diluted to 100 cc. with water.

CYANOGEN BROMIDE SOLUTION

This was prepared by decolourising in the cold a saturated solution of bromine water by the careful addition of 10 per cent potassium cyanide. The solution was kept in an ice bath at 4-5°.

Colour reaction.—Standard nicotinic acid solution or a known amount of the test solution (adjusted to *p*H 5-6) was taken in a series of test tubes with a 15 cm. mark. The solutions were diluted to 10 cc. with water and then heated in a water bath at 75—80° for about 5 minutes. One cc. of cyanogen bromide solution was then added to each and the mixtures were heated again for 5 minutes at 75—80°. The test tubes were then cooled in an ice bath for 5 minutes. One cc. of the amine reagent was now added to each and the volume of the solution was made up to 15 cc. with distilled water. These were kept in the dark for 5 minutes for the full development of colour. The colours were compared within 15 minutes in a colorimeter.

This procedure was found to give accurate results using different quantities of Brewers yeast and a number of other natural materials. The recovery of pure nicotinic acid added to any test materials was almost 100 per cent. Tables I and II are illustrative of the results obtained.

TABLE I.

Estimation of nicotinic acid in yeast.

No.	Amount* of yeast taken (mg)	Colorimeter reading with 40 μg* nicotinic acid as standard kept at 20°.	Nicotinic acid found, Micrograms per g.
1.	50	34.5	469.0
2.	60	29.0	470.0
3.	70	25.3	458.0
4.	80	22.0	455.0
5.	90	19.8	449.0
6.	100	17.4	460.0

TABLE II.

Recovery of added nicotinic acid from yeast.

No.	Amount* of yeast taken (mg)	Amount* of nicotinic acid added (μg)	Amount of nicotinic acid found (μg)	Amount of nicotinic acid theoretically present.	Recovery Per cent.
1.	50	0.92	44.1	43.0	102.6
2.	50	1.84	62.1	63.0	98.6
3.	50	2.76	84.1	83.0	101.3
4.	50	3.68	102.3	103.0	99.3
5.	50	4.60	123.3	123.0	100.2

*Larger quantities were weighed each time and made into solution and proportionate amounts taken.

A few precautions are necessary to obtain accurate results. Standard solutions should not be kept longer than 3—4 days. Cyanogen bromide solution should be freshly prepared not more than half an hour before its use and the solution of bromine in water used, should be just saturated, not containing any excessive amounts of bromide. It is also necessary to adjust the *p*H to 5—6. The solution must be heated before adding the cyanogen bromide solution to get full development of the colour in the subsequent procedure.

The colour by the procedure prescribed develops fully in less than 15 minutes. The method is therefore advantageous as compared to that of Bandier and Hald (4) which require one hour for the maximum development of colour. The colour developed by this method is brilliant yellow much more intense than obtained by Swaminathan's or Bandier and Hald's methods, and is only slightly less intense than that obtained by the method of Harris and Raymond. The stability of the colour is much greater than in any of the other three methods.

A blank is necessary only when the test solution itself is sufficiently coloured to cause a significant error.

Summary

A critical study of the cyanogen bromide method was made using different amines. A modified procedure is described involving the use of aniline in the form of its hydrochloride which has been found to give the best results—giving an intense colour stable for over 30 minutes. A study has also been made of the various factors e.g., *p*H, presence of inorganic salts and organic compounds, temperature. variations in the quantity of reagents, affecting the colour reaction and a procedure is described which yields accurate and reliable results.

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ON THE ANTIBACTERIAL ACTIVITY OF SULPHA DRUGS AGAINST BACILLARY DYSENTERY ORGANISMS

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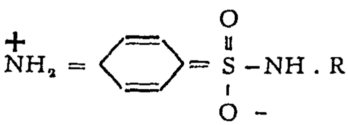
(Received for publication, April 3, 1946)

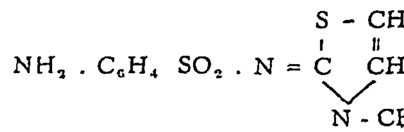
Our chemotherapeutic investigations with sulphanilylbenzamide led to the preparation of two isomers *p*-ethylaminobenzene-sulphonbenzamide (II) (Basu and Sikdar, 1). The compounds are soluble in alkali, and form sodium and silver salts, but do not undergo any further reaction with an alkyl iodide indicating thereby that the imide hydrogen atom as present in the above compound (II) is fundamentally different from the hydrogen atom as present in the mother compound—sulphanilylbenzamide (1). This chemical inactivity of the compound led us further to study its bacteriostatic property and it is now being found that it is not also active bacteriologically (*vide infra*). This suggests that in order to exert its characteristic bacteriostatic activity the compound must possess a free *p*-amino grouping. Of course compounds are now known (Goetchins and Lawrence, 2) which are found to be active inspite of the absence of such an amino group. But according to the mechanism of the action of sulphanilamide type compounds as proposed by Woods and Fildes (3) the therapeutically active compounds compete for the essential metabolite *p*-aminobenzoic acid. The reactions of enzyme systems are again essentially chemical. As such any change brought out in the *p*-amino grouping of a sulphanilamide type compound may alter its physiological characferistics along with the physical properties. Kumler and Daniels (4)

suggest that a fundamental factor for activity is the contribution of the resonating form with a coplanar group (III) (*cf.* Bell and Roblin, 5).

$NH_2\ C_6H_4\ SO_2\ NH\ CO\ Ph$
(I)

$Et\,.\,NH\,.\,C_6\ H_4\ SO_2\ NH\,.\,CO\ Ph$
(II)


(III)


(IV)

$NH_2\,.\,C_6\ H_4\,.\,SO_2\,.\,CH_3$
(V)

$NH_2\,.\,C_6\ H_4\ SO_2\,.\,CH_2\,.\,CO\,.\,CH_3$
(VI)

If R in the form (III) be an electron repelling, it would differ from the compound where R is electron attracting. As in the former case the charge on the amide nitrogen would be altered with the consequence that the ionisation of N′-hydrogen atom would be lowered down, and the compound would become less soluble in hydrated solvent and at alkaline *p*H. It may be expected that in order to block the enzyme systems that might be present during any bacterial disease the chemotherapeutic compound should be present both in ionic as well as in undissociated molecular form so that it may penetrate through all the cellular tissues of the host and the organism (*cf.* Clowes and Keltch, 6 ; Lwoff *et al,* 7). This, is somewhat contradictory to the general belief that for therapeutic action against intestinal organism the compounds are to be comparatively insoluble and poorly absorbed from the gut. Of course the activity of sulphaguanidine, succinyl sulphathiazole and phthalyl sulphathiazol are due to the above factors. But recent observations of Bose and Ghosh (8) on the activity of sulphanilyl benzamide against bacillary dysentery organism tend to show that better results may be obtained from a drug that would maintain an effective concentration in blood as well as in the intestinal contents. For this the drug must be present both in ionic as well as in undissociated molecular form as it is the latter that can penetrate through the membrane of the cells of the organism. (Hardy and Watt, 9 ; White *et al* 10). This seems to be more probable in a dysentery infection. In bacillary dysentery cases two problems may arise depending upon the severity of infections. One is concerned with the treatment of mild cases when the bacteria may reside chiefly in the lumen of the intestinal tract and the other case may arise when the pathogenic bacteria may be present not only in the lumen of the intestinal tract but also in the living tissue often covered by muco-purulent exudate as in severe infections. Work is in progress to correlate the relative dissociation, absorption and therapeutic activity of certain sulphanilamide type compounds. The present paper describes only the *in vitro* activity of certain sulpha compounds (Table I) against bacillary dysentery organism in relation to their acid dissociation constants. The Table indicates the milligram of the drug per 100 cc. of culture medium made from papain-digest glucose phosphate meat broth that was necessary for inhibiting the growth of the dysentery organism when incubated at 37° for 72 hours. The acid dissociation constants were recorded from purified sulpha compounds.

TABLE I

Organism used:	Bacillary dysentery, Flexner Y.
Number of organisms:	1000 per 5 cc.
Period of incubation:	72 hours.

Figures under column (*a*) indicate milligram of drug per 100 cc. bringing forth bacteriostasis; and figures under column (*b*) indicate the *pka* value as a measure of the acid dissociation constant.

DRUG	(a)	(b)
Sulphanilyl benzamide (I)	10	4.27
Acetyl ,, ,,	> 100	—
Sulphanilyl acetamide	50	5.45
Ethyl sulphanilyl benzamide (II)	> 100	5.05
Sulfathiazole	2	7.15
Methylated sulfathiazole (IV)	10	8.7
p-Amino phenyl methyl sulphone (V)	100	11.0
p-Amino phenyl-aceto methyl sulphone (VI)	20	9.2

From the table it is evident that the *in vitro* activity bears a close relationship with the acid dissociation constant of the compound. It is known that in the case of methyl derivation of sulfathiazole—(2-sulfanilimido-3-methyl-2: 3-dihydrothiazole, IV) the activity of the parent compound sulfathiazole is retained to a considerable extent in its action against *E. Coli* whereas the methylation of the imide hydrogen with the formation of the compound N′-methyl sulfathiazole practically annuls the activity of the thiazole compound (*cf.* Shepherd, Bratton and Blanchard, 11). The N′-methyl compound was not in our hands and as such could not be compared but the above dihydro-thiazole (IV) was prepared by treating a suspension of silver salt of sulfathiazole in benzene gradually with methyliodide dissolved in benzene, for more than an hour on a boiling water bath. The mixture was filtered hot, the precipitate washed with dry benzene, and refluxed with alcohol. The alcoholic extract was filtered and cooled. The methylated sulphathiazole (IV) separated out and was crystallised from alcohol (95 per cent) in microscopic needles, m.p. 250-51°. The methylation lowered the acidity of the compound and with that its *in vitro* activity to a considerable extent.

It may be further noted from the table that the *p*-aminophenylmethylsulphone (V) which is practically a basic compound exerts no *in vitro* activity. The substitution of the hydrogen atom by one electron attracting group (acetyl) in *p*-aminophenylacetylmethylsulphone (VI), however, increases its acidity and its antibacterial activity *in vitro*. Apart from the chemical properties which a drug may possess, its physical properties may play a role in its therapeutic value (*cf*, Gilligan, 12).

SUMMARY

The *in vitro* activity of certain sulpha drugs against bacillary dysentery organism (Flexner Y) along with their acid dissociation constants was studied. It appears that the lowering of the acidity of a sulpha compound bears a direct relationship with its antibacterial activity *in vitro*. It is suggested that although the chemical nature of the drug itself is mainly responsible for physiological activity, the physical properties such as its dissociation in body fluids, and permeability through the cell membranes may play a considerable part in determining its chemotherapeutic action. Further work in this direction is in progress.

In conclusion, the authors wish to express their thanks to Mr. R. P. Banerjee for help in ascertaining the acid dissociation constants.

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CHEMOTHERAPEUTIC STUDY OF PNEUMOCOCCAL INFECTIONS

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(Received for publication, April 2, 1946)

While investigating the physiological characteristics of sulphanilyl benzamide—a compound that is being found in our hands to be an effective antibacterial agent in bacillary dysentery infections (1, 3)—it was noticed that the compound also possesses a definite anti-pneumococcal activity (2). As the alteration of the *p*-amino group in a sulphanilamide type compound to amidine is being found (4, 5) to widen the chemotherapeutic activity in sulphonamide compounds, it was considered to be of interest to study the anti-pneumococcal activity of the compound that might be obtained by replacing the amino-group of sulphanilyl benzamide (I) by amidine. The compound *p*-amidinobenzene-sulphon benzamide (II) has been prepared and described by Sikdar and Basu (6). The product used in this investigation was the hydrochloride, m.p. 210-211°. Its mother compound *p*-amidinobenzenesulphonamide hydrochloride (III), m.p. 242-44°, was studied against experimental gas gangrene by Evans *et al* (5). As the previous authors also noted an enhanced activity in *p*-amidinophenyl- (IV) as well as *p*-aminomethylphenyl methylsulphone (V), they were all studied in the present investigations. The latter compound was found to be the most active *in vitro* against pneumococcus. The allied sulphones *p*-aminomethyl phenyl-acetomethyl sulphone hydrochloride (VI), m.p. 208°, and methylene bis-(*p*-aminomethylphenyl sulphone)-dihydrochloride (VII) were found to be practically inactive.

It may be pointed out here that the sulphonamide corresponding to the compound *p*-aminomethylphenyl methylsulphone under the name of 'Marfanil' has been found to be a very useful drug in the treatment of infected wounds particularly for highly purulent cases of chronic sepsis (7). This compound

has been previously found by Basu *et al* (9) to be definitely bacteriostatic against *Vibrio Cholerae* (types: Inaba and Ogawa). All these led us to study the bacteriostatic activity of the various amidines and sulphones as mentioned above against *strepto*-and *staphylococcus* organisms. In this study too the compound *p*-aminomethylphenyl methyl sulphone was found to be the most active agent. In view of this characteristic *in vitro* activity, the protective action of this compound against pneumococcal infection (type I) in mice was studied. The results of all these investigations are being recorded in the body of this paper.

$NH_2\,C_6H_4\,SO_2\,NH\,CO\,Ph$ (I)

$(NH)(NH_2)\!>\!C\,C_6H_4\,SO_2\,NH.\,CO\,Ph$ (II)

$(NH)(NH_2)\!>\!C-C_6H_4\,SO_2\,NH_2$ (III)

$(NH)(NH_2)\!>\!C-C_6H_4.\,SO_2\,CH_3$ (IV)

$NH_2\,CH_2.\,C_6H_4\,SO_2\,CH_3$ (V)

$NH_2\,CH_2\,C_6H_4\,SO_2\,CH_2\,CO\,CH_3$ (VI)

$NH_2\,CH_2.\,C_6H_4\,SO_2\,CH_2-CH_2-SO_2\,C_6H_4\,CH_2\,NH_2$ (VII)

Experimental

Antibacterial Activity in vitro:—The compounds in the form of their hydrochlorides were used. The usual process and technique were followed. Table I shows the minimal concentrations, expressed in mg. of drug per 100 cc. of culture medium (made from papain-digest-glucose phosphate-meat broth) which prevented the appearance of visible growth during 72 hours at 37°. Each recorded value is the result from an average of three tests. The number of organisms used were about 1000 colonies per 5 cc. of broth.

TABLE I.

Organism	I	II	III	IV	V	VI	VII
Strepto. hæmolyt.	10	100	2	...	1	...	100
Strepto. viridan	10	100	...	..	1	...	...
Staph. aureus	100	100	...	...	2	100	100
Pneumo. Type I	2	100	10	2	1	nil	100
,, Type II	10	100	20	10	2	nil	100
,, Type III	10	100	20	10	2	nil	100

From Table I it is evident that compound (V) *p*-aminomethylphenyl methylsulphone is the most active agent. As such its action against pneumococcal infection in mice was studied.

Chemotherapeutic Action in vivo:—The strain of pneumococcus used was a virulent, frequently passaged strain of pneumococcus type I. Approximately 0.2 cc. of 10^{-5} dilution of an 18-hrs' hormone broth culture produced a fatal infection in mice. The animals weighing 20-22 g. were used throughout and the technique employed in assessing the therapeutic activity of the drug (V) was based on the drug diet technique, as well as that described by Bose, Das Gupta and Basu (3). The protection tests were carried out with fairly high infecting doses of organisms, and positive controls with sulphapyridine were always kept with negative controls in each set of experiments. Treatment was done both by oral feeding, as well as by parenteral administration. The results of the investigations are recorded in Table II.

TABLE II.

Showing the action of *p*-aminomethylphenyl methylsulphone against virulent pneumococcal Type I infections in mice. All animals infected with a dose of 0.2 c.c. of 10^{-5} dilution of 18 hours hormone broth culture of pneumococcus type I.

Drug-diet therapy started one day previous to infection. Other mode of therapy starting on the same day as infection. Weight of mice 18-22 gms. Treatment continued till death.

Drug	Therapy	No. of mice	Survival after 14 days	Average survival time (days)	Control mice with each expt. (without drug)	
					Mortality	Average survival time (days)
Marfanil	1. Subcutaneous 0.25 mg./g.	10	0	2.0	5/5	1.0
	2. In diet 0.5%	10	0	1.2	5/5	0.95
Sulphone compound (V)	3. Subcutaneous 0.5 mg./g.	10	0	1.0	5/5	1.0
	4. In diet 0.5%	10	0	1.2	5/5	1.0
	6. In diet 1%	10	0	1.8	10/10	1.0
Sulphapyrdine	7. In diet 0.5%	10	9	12.8	5/5	1.0
	1.0%	10	10	14.0	10/10	1.9

SUMMARY

In vitro activity of certain sulphonamide and sulphone derivatives was studied. The promise shown by *p*-aminomethylphenyl sulphone in its activity against pneumococcus, was not fulfilled from its protective action in mice against pneumococcal infection. 'Marfanil' (*p*-aminomethyl benzene sulphonamide) has no protective action on mice against pneumococcal infection.

In conclusion the authors wish to express their sincere thanks to Dr. U. P. Basu for his suggestions in the course of the investigations.

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ADVANCEMENT OF KNOWLEDGE
INDIAN INSTITUTE FOR MEDICAL RESEARCH
SERVICE TO MANKIND

PUBLISHED QUARTERLY UNDER THE AUSPICES OF THE INDIAN INSTITUTE FOR MEDICAL RESEARCH, CALCUTTA.

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Annals of Biochemistry and Experimental Medicine

Volume V, Number 4, 1945

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HALOGENATED ORGANIC INSECTICIDES PART I

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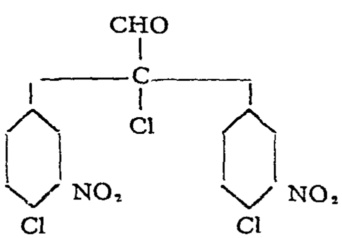
The success which has resulted in the employment of D.D.T. *pp*-dichloro diphenyl trichloroethane) as an insecticide and bactericidal agent has opened up a line of investigation about synthetic organic insecticides. Zeidler (1) was the first to record the condensation of chloral and chlorobenzene but the value of the product as an insecticide was not realised at the time. In the course of the second world war when the supply of pyrethreum and rotenone was cut off, attention was directed to the search of synthetic insecticides and in the course of this, Zeidler's preparation was found to be a very efficient one. It has been claimed that D.D.T. was remarkably useful in bringing under control a severe out-break of typhus in Naples after the occupation of that city by the Allied forces. In India, the compound has been extensively used for anti-malarial work by the U.S. Army Medical Units. In the early part of 1945, it was estimated by competent authorities that the yearly requirement of D.D.T. for the army would be about 5000 tons. As there are no local sources for the manufacture of chloral and chlorobenzene, the demand for the armed forces would be met by import.

In the present communication, the preparation and properties of the compounds of the type of D.D.T., where the nuclear chlorine atom has been replaced by other halogens, have been described. The condensation of *o*-nitro-chlorobenzene and chloral gave very curious results. When the reaction was carried out according to Zeidler's method, a product was obtained with a m.p. 114°C. But the analytical data did not correspond with any known compound and the m.p. is also different from that of chloral hydrate. The substance was found to be nitrogen-free and its constitution has not yet been finally settled. When, however, the same reaction was carried out with fuming sulphuric acid, a product with m.p. 85°C was obtained. The analytical data suggest the following structure which is supported by the formation of a semi-carbazone.



It has been noticed in the course of this work that copious evolution of acid fumes takes place during the condensation. If the reaction is limited to the elimination of a molecule of water from the two para-hydrogen atoms and the

aldehydic oxygen, the evolution of hydrochloric acid gas is somewhat curious. But as it appears in the case of *o*-nitro chlorobenzene, if the reaction consists in the elimination of two molecules of hydrochloric acid, the evolution of acid fumes is easily explained. It thus appears that two reactions take place simultaneously—(*i*) the elimination of two molecules of hydrochloric acid and (*ii*) the elimination of a molecule of water. In the case of simple halogen substituent products, the second reaction is the predominating one while in the case of *o*-substituted derivatives the main reaction takes place according to the first scheme. It is also somewhat curious that *o*-chloro-benzoic acid does not condense, as the unchanged starting material is obtained. Acetanilide also fails to react, probably on account of the non-reactive character of the *para* hydrogen atom.

From some experiments carried out to determine the toxic effectiveness of these compounds on *Anophaelis larvæ* as compared with D.D.T., it is clear that the nuclear halogen atoms are not of any great importance and the activity of this type of compounds depends upon the group$=CH.C.Cl_3$ joining the phenyl radicles. In future communications, the following points will be taken for consideration:

(*i*) Increasing the number of nuclear halogen atoms.
(*ii*) Replacement of the three chlorine atoms in side chain by bromine and if possible, with iodine.
(*iii*) Replacement of benzene nucleus with napthalene or anthracene.

Experimental

Condensation of Chloral and *Iodo-benzene*:—Chloral (15 g.), iodo-benzene (41 g.), sulphuric acid (95%, 95 cc. or 185 g.) and fuming sulphuric acid (20% SO_3, 60 g.) were mixed in a three-necked round-bottom flask (1000 cc.) fitted with a mercury-sealed mechanical stirrer, a condenser with guard-tube and a thermometer. The mixture was stirred vigorously for about 4 hours, the temperature being maintained between 50°-60°C. A red plastic mass separated which together with the acid liquid was poured over crushed ice and the whole left in a refrigerator overnight. The solid was filtered off and the mass washed thoroughly with water. The solid was taken in a beaker and hot water was poured into it and the mixture stirred vigorously. The clear liquor from the top was decanted off. This was repeated thrice. Subsequently the mass was treated with dilute sodium bicarbonate and sodium thiosulphate solutions respectively. It was then filtered, washed and pressed between filter papers to remove the adhering oil. The whole was then crystallised from rect. spirit (charcoal), m.p. 165°-167°C. It was again crystallised from acetic acid in white glistening needles, m.p. 167-C. Yield of recrystallised product—20 g.

(Found: C-31.86%, H-2.13% ; $C_{14}H_9Cl_3I_2$ requires C31.07%, H-1.67%)

Condensation of *Chloral* and *Bromobenzene*:—Chloral (15 g.), bromobenzene (31 g.), sulphuric acid (95%, 350 g.) and fuming sulphuric acid (20% SO_3, 50 g.) were treated in the same manner for 4 hours. The solid was filtered off, washed with hot water to remove absorbed acid. The crude product was suspended in hot water and mechanically stirred to remove any excess of chloral, then pressed between filter papers and crystallised from rect. spirit, m.p. 141°C.

(Found C-38.60%, H-2.35% ; $C_{14}H_9Cl_3Br_2$ requires C-37.92%, H-2.70%)

Condensation of *Chloral* and *o-Nitro-chlorobenzene in presence of conc. sulphuric acid*:—Chloral (20 g.), *o*-nitro-chlorobenzene (10 g.) and sulphuric acid

(sp. gr. 1.82,120 cc.) were mixed together in 500 cc. conical flask and shaken occasionally. The mixture was warmed on a water-bath from time to time. The reaction was allowed to proceed for 4 days. At the end of this period the acid liquor together with the separated solid was poured over crushed ice. A brown pasty mass was obtained which was vigorously agitated with hot water to remove chloral and absorbed acid, then filtered and pressed. The mass was then crystallised from rect. spirit (charcoal) and obtained in white glistening needles, m.p. 114°C. (Found C-19.20%, H-0.98%). The substance was nitrogen-free.

Condensation of Chloral and o-Nitro-chlorobenzene in presence of fuming Sulphuric acid.—Chloral (30 g.), *o*-nitrochlorobenzene (63 g.), sulphuric acid (95%, 190 cc.) and fuming sulphuric acid (20% SO_3, 100 g.) were mixed in a three-necked round-bottom flask fitted with a condenser, a thermometer and a mercury-sealed stirrer. The contents of the flask were vigorously stirred for 6 hours at a temperature of 66°-70°C. The colour of the liquid changed to red and copious acid fumes were given off during the operation. The acid liquid together with the separated solid was poured over crushed ice and left in a refrigerator overnight. The solid was filtered off, washed with water and dried between sheets of filter paper. The crude mass was then crystallised first from dilute alcohol and then from dilute acetic acid, m.p. 85°C.

(Found: C-43.70%, H-2.54%, N-7.63%, Cl-27.31% ; $C_{14}H_7O_5N_2Cl_3$ requires C-43.20, H-1.81%, N-7.20%, Cl-27.20%).

The substance was insoluble in water, acids and alkalies. The analytical data suggest the structure given before and this was supported by the formation of a semi-carbazone, which was crystallised from dilute methyl alcohol, m.p. 168°C. (Found N-15.62% ; $C_{15}H_{10}O_5N_5Cl_3$ requires N-15.71%).

Experiments to determine the relative toxic effectiveness on anophelis larvæ of some compounds as compared with DDT.—These tests were conducted by placing an equal number of anophelis larvæ in white enamelled pans, containing deep well water. Treatment was then applied by adding 5 drops of solution in the respective pans. The drops of solution were released from a hypodermic syringe with a needle, No. 25 ; this gives approximately 200 drops per cc. The drops were released at about one foot above the water. The area of the water is about 1.2 square feet.

At various subsequent time intervals the number of larvæ still floating on the surface of the water were counted and recorded. The larvæ that are affected fall to the bottom and squirm. A few minutes after they fall they have extreme difficulty in reaching the surface again, and their condition becomes progressively worse as the minutes go by. The majority of them are usually knocked down in one to two hours, and are finally dead after 6 to 10 hours.

The results are shown in the following tables:—

June 25, 1945—10.30 A.M.
Kerosene solutions (5% w/v)
Larvæ left floating.

Hour	10.30	11.30	1.30	2.30
DDT	70	70	37	9
N-1	70	70	42	4

July 4, 1945—11.30 A.M.
Kerosene solutions (5% w/v)
Larvæ left floating.

4.00 slightly more	Hour	11.30	12.30	2.00	4.00
Those on bottom at	DDT	60	3	0	0
active.	N-2	60	13	3	1

July 7, 1945—11.30 A.M.
Kerosene solutions (5% w/v)
Larvæ left floating.

Hour	11.10	12.30	2.30
DDT	50	0	0
N-3	50	26	9

July 7, 1945—2.45 P.M.
Kerosene solutions (5% w/v)
Larvæ left floating.

Hour	2.45	3.30	4.10
DDT	50	5	0
N-2	50	12	4

August 29, 1945—3.00 P.M.
Xylene solutions (5% w/v)
Larvæ left floating.

Hour	3.00	4.00	4.30	5.00
DDT	50	40	13	3
		10	24	34
		pupae	pupae	pupae
N-4	50	46	24	21
		9	22	25
		pupae	pupae	pupae
Knocked down :				
	50	10	13	13
	50	4	4	4

August 31, 1915—11.20 A.M.
Xylene solutions (5% w/v)
Larvæ left floating.

Hour	11.20	12.20	1.20
DDT	60	30	15
N-4	60	60	60

N-1.—Dichlorodiphenyl trichloro-ethane.
N-2.—Dibromo-diphenyl trichloro-ethane.
N-3.—Di-iodo diphenyl trichloro-ethane.
N-4.—bis (3-nitro 4-chloro-phenyl) mono-chloracetaldehyde.

The investigation was carried out under the auspices of the Adair Dutt Research Fund Committee. The author's grateful thanks are due to Dr. S. Niyogi for his kind interest in the work and helpful criticism. Thanks are also due to Mr. N. Ghose for the micro-analysis of the substances and to S/Sgt. H. S. Chubbs of the U.S. Army for the physiological tests.

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SOME OBSERVATIONS ON THE SYNTHESIS OF PROTEINS BY PLANTS

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Russel (1) is of opinion that nitrogen must be presented to green plants in the form of nitrate in order that it may be further synthesised into a form suitable for the needs of animals and atmospheric nitrogen is useless for the purpose. The same idea has been put forward by Bayliss (2) thus, "The green plants need no further nitrogenous food than nitrates so that the constituents of its proteins which are identical with those of animal protein must be formed in the organism itself." Howell (3) also says that the plant organism utilises inorganic forms of nitrogen such as ammonium salts or the nitrates in building up its proteins. So in order to ascertain how far the soil nitrogen is responsible for the formation of proteins by green plants, the present work was taken up.

Experimental

Bengal gram. (*Cicer arietinum*) was taken as seeds for growing plants. The percentage of nitrogen in dry grams was estimated and thus the protein content was ascertained by taking the mean of five estimations. Out of this stock 150 g. were taken and divided into 75 equal parts of 2 g. each. All of them were put in water in porcelain basins separately ; water was drained out from 50 of these basins after 24 hours and the samples were spread out on damp earth and covered over with pieces of moist cloth kept moist by sprinkling water from time to time so as to allow them to germinate (McCarrison, 4). The remaining 25 samples were kept immersed in water throughout the experimental period. On the third day the fomer were uncovered and were all found in a sprouting condition. Twenty of them were planted in pots measuring 36 square inches in area in a soil taken from a garden (previously manured with cattle manure) and the remaining 20 in another series of pots of same size containing a soil manured with potassium nitrate, and the plants were allowed to grow in them. On every fourth day some samples (usually five) were taken out from each of the four groups viz, (*i*) immersed in water, (*ii*) sprouting, (*iii*) growing in cattle-manured soil, and (*iv*) growing in nitrated soil, the last two being washed carefully so as not to lose any part, the leaves, the stems, or the roots. The nitrogen content and the protein content too were ascertained by taking mean of five readings in each case, by the standard Kjeldahl's method. The percentage of nitrogen of the soil too in the pots from which the plants were taken out was also estimated on every fourth day so as to find out the loss of nitrogen, if any, utilized by the growing plants.

Table I shows the percentage of nitrogen in the gram (*i*) immersed in water (*ii*) sprouting (*iii*) grown on cattle-manured soil and (*iv*) nitrated soil, every fourth day for two weeks.

TABLE I

*Showing percentage of Nitrogen.**

	In water	Sprouting.	Grown on C-M. soil.	Grown on N. soil.	In C-M. soil.	In N. soil.
1st Day	3·0079	3·0073	...	...	...	...
4th Day	3·10312	3·1948	3·19482	3·19482	0·12537	0·1645
7th Day	3·19701	...	3·23574	3·2556	0·11088	0·1539
10th Day	3·17394	...	3·465	3·2553	0·112	0·1512
13th Day	3·10859	...	3·2725	3·2632	0·1032	0·1478

* The percentage of proteins in grams is found out by multiplying the percentage of nitrogen by 6·25.

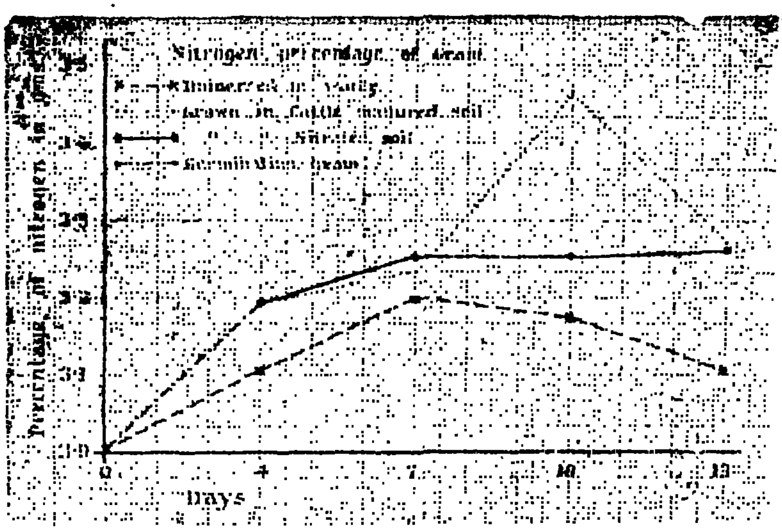

CHART I.

From the above table and the accompanying charts it is well evident that when gram seeds are immersed in water the percentage of nitrogen and hence of protein rises to some extent till the 7th day and then declines gradually till the 13th day. When they are allowed to germinate (4th day) the content is about 1 p.c. higher than when they are put in water without germination. Next when the sprouting seeds were transferred to the soil the percentage of nitrogen (hence of protein too) goes higher up to 3·46 p.c. on the 10th day (initial one being 3 p.c.) and then comes down again gradually to about 3·2 p.c. on the 13th day. Along with this increase in the percentages of nitrogen and protein in the sprouting gram, there is only slight loss of nitrogen (about 0.012 p.c.) on the 4th day after which the soil retains its nitrogen almost at the same level till the 13th day. The sprouting gram in the nitrated soil, however, has just the same increase in the percentages of nitrogen and protein as those grown in the cattle-manured soil, but later on had constant amounts of nitrogen and protein till the 13th day without any further increase. The nitrated soil too lost 0·012 p.c. of nitrogen on the 4th day but the percentage of nitrogen in the soil manured with nitrate remained almost constant later on till the 13th day.

In this respect the condition of the cattle-manured soil ran almost parallel to that of the nitrated soil when plants were grown in it (Chart II).

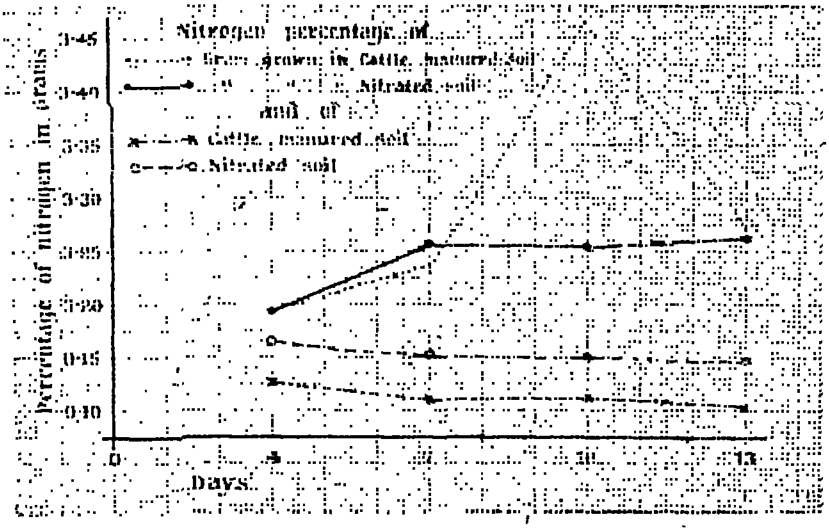

CHART II.

Discussion

From the above findings we conclude that there is always some gain in nitrogen content (and hence protein too) of the gram seeds when they are immersed in water or are allowed to germinate on the 3rd or 4th day. There is still further gain up to the 7th day, whether the seeds are immersed in water or they are sown in cattle-manured or nitrated soil. Later on there is a steady decline in the percentage of nitrogen as also that of protein till the 13th day in the case of grams still immersed in water. Those that were sown in cattle-manured soil had still further increase in the nitrogen content (and consequently that of protein too), on the 10th day and then the curve had a downward course till the percentage was about the same on the 13th day as on the 7th day. On the other hand those that were grown on the chemically manured soil scarcely showed any increase in nitrogen or protein content and maintained a more or less constant level from the 7th to the 13th day.

The upward tendency of the curves of the percentage of nitrogen in plants growing in cattle-manured and nitrated soil from the 4th to the 7th day can be easily explained by assuming that the plants derived some of their increase in nitrogenous content from the soil as is evident by the simultaneous downward curves of the soil nitrogen for both cattle-manured and nitrated soil. But further upward deviation of the nitrogen curves of plants grown in the former soil on the 10th day or again coming down to the former level on the 13th day cannot be explained so easily, as the nitrogen curve of the cattle manured soil remained at a constant level all throughout without any downward tendency. Curiously enough, the curve of nitrogen of plants growing on nitrated soil also remained at a constant level showing thereby no depletion of the soil nitrogen to the plants from the 7th to the 13th day—which is corroborated by the curve of soil nitrogen of the chemically manured soil, running a parallel course with the plant nitrogen curve. These observations throw much doubt on the assertion made by Bayliss (2) that green plants need no further nitrogenous food than nitrates. Viswanath and Suryanarayana (5) also found that

mineral manure has not a very good cropping value and is in this respect much inferior to cattle-manure. This has also received further corroboration by McCarrison's observations (6) that 'cattle-manure' wheat was of more nutritive value than 'chemical manure' wheat.

But neither the upward curve of the nitrogen content of plant grown on cattle-manured soil from the 7th to the 10th day, nor the upward curve of nitrogen curve of germinating gram or that immersed in water can be explained by bringing the question of nitrification of the soil. Some other factor must be brought in to explain these. They can only be explained if we trace the source of gain in nitrogen to the atmospheric nitrogen. That this is possible has been proved by Beijernick (7) who showed that bacteria of the soil are able to obtain nitrogen from the atmospheric nitrogen. This explains the upward tendency of the curve of the plant nitrogen grown on cattle-manured soil but why that comes down again can not be explained. Moreover the gain in the nitrogen content of the germinating gram or gram immersed in water without germination for the first three days, too cannot be similarly explained. Surely the bacteria cannot play their part in these cases. So some other agencies for the depletion of nitrogen from the atmosphere have yet to be found out for the full explanation of all the observations detailed above.

SUMMARY

(1) Sprouting Bengal gram or gram soaked in water for a few days has more protein than dry gram.

(2) The gram plants derive the gain in nitrogen from that of the soil. In this respect the mineral manure is less efficient than cattle manure.

(3) The increase in the protein content of the plants cannot be explained entirely by depletion of nitrogen from the soil unless part of it is derived from the atmospheric nitrogen by the help of the soil bacteria.

(4) The atmospheric nitrogen is alone responsible for the increase in the percentage of protein in the germinating gram and also that immersed in water. How this takes place has yet to be explained.

(5) In the case of nitrated soil the percentage of plant nitrogen did not rise at all after 7th day, possibly because nitrates somehow or other interfered with the fixation of nitrogen from the atmosphere by the bacteria (Waksman, 8). When this is the case, Abderhalden and Rona (9) advocate addition of little cane sugar to the soil to make the inactive bacteria active again. So also Dhar (10) has shown that addition of some molasses facilitates nitrogen fixation appreciably in plots and dishes containing nitrogenous substances.

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THE ROLE OF PHOSPHORUS IN THE ANIMAL ORGANISM. PART I. INORGANIC PHOSPHORUS CONTENT OF WHOLE BLOOD, CELLS AND PLASMA IN HEALTH AND DISEASE.

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For the diagnosis and prognosis of rickets, osteomalacia, osteoporosis, and other skeletal diseases, the pathological changes in the blood constituents have been investigated by a number of workers in this field. These for the most part have remained limited to the study of blood serum calcium, phosphorus and phosphatase. At the same time in view of the fact that the type of rickets commonly found in Europe and America, is due to low phosphorus and high calcium diet, the studies carried out on the experimental animals have largely related to high calcium and low phosphorus diets and the blood serum analyses have remained limited to the determination of inorganic phosphorus in the blood serum or plasma. Relatively few workers have paid attention to phosphorus in the cells or investigated the question whether cellular phosphorus had any rôle to play in phosphorus metabolism under such conditions.

Brain, Kay and Marshall (1), Stearns and Warweg (2), Barrensheen, Doleshall and Popper (3), Snyder and Katzenelbogen (4) have all stated that the quantity of inorganic phosphorus present in blood is greater in the plasma than in the cells.

Buell (5) could not find any significant amount of inorganic phosphorus in the cells and whatever little was traced, was believed to be due to enzymic hydrolysis of the organic phosphorus compounds. On the other hand Bloor (6), Freudenberg (7), Stanford and Weathley (8), found inorganic phosphorus concentration in the cells to be higher than in the plasma or in the whole blood. More recently Behrendt (9), by following the method of fractional hydrolysis of the whole blood, and by heating the trichloracetic acid filtrate of the whole blood for various intervals of time, according to the technique of Lohmann (10) found that the concentration of inorganic phosphorus in the cells was equal to or even slightly higher than that in the plasma. This confirmed the observations of Euler and Myerback (11) that for guinea-pigs and rats 42 per cent of inorganic phosphorus of the whole blood was on the average present in the red cells.

In view of these somewhat contradictory results, it was decided to study the relationship under normal conditions between inorganic phosphorus in the red cells and in the plasma or whole blood. For this study healthy students, and members of the staff of the Punjab University Institute of Chemistry and patients attending the medical outdoor department of the Mayo Hospitals Lahore for minor ailments were selected.

The study has been extended to include the determination of inorganic phosphorus in the blood cells and plasma in a number of pathological conditions. In case differences were observed it was intended to investigate if the inorganic phosphorus in the blood serum, whole blood or the cells had any relationship with (a) the nature of disease, and (b) with the severity of the condition.

Method

For the determination of inorganic phosphorus one cc of whole blood, or one cc. serum or plasma was taken and precipitated with 9 cc. of 10 per cent trichloroacetic acid and left in the ice chest for fifteen minutes. When examining whole blood it was treated with 10 per cent trichloroacetic acid within one minute of withdrawal. It was found that the small amounts of oxalate required to check clotting in the case of plasma did not interfere with phosphorus determinations. After 15 minutes each sample was removed from the ice chest and filtered through Whatman No. 44 ashless filter paper. In the filtrate the determination of inorganic phosphorus was made according to a modified method of Holman (13) comparing the colours in a Hellege Dubosq Colorimeter.

Inorganic phosphorus in the cells was calculated from the values of the whole blood and plasma or serum using the equation

$$X = Y - \frac{100\,(Y - Z)}{V}$$

where X represents the inorganic phosphorus in the cells,

	Y	do	do	plasma or serum,
	Z	do	do	whole blood,
and	V represents the volume of the packed cells.			

The volume of the packed cells was determined by the hematocrit tube method using graduated tube of 5 cc capacity with 100 markings, centrifuged at 2500 r.p.m. for 30 minutes.

Normal Cases :

The results of the examination of the blood of 30 normal adult males, 10 females, and 10 children are summarized in Table I.

TABLE I

Inorganic phosphorus content of blood in normal persons.

No.	Age of the subject in years.	Volume of packed cells. %	mg. Phosphorus per 100 cc.			
			Whole blood.	Serum.	Plasma.	Cells.
Adult Males.						
1.	14	39	2.40	3.9	...	0.06
2.	23	37	2.85	...	3.8	...
3.	35	37	2.40	...	3.5	0.50
4.	21	38	2.20	3.4	...	0.25
5.	55	39	2.52	1.0	...	0.21
6.	18	38	2.35	...	3.5	0.48
7.	48	37	2.10	3.25	...	0.15
8.	16	38	2.21	...	3.42	0.21
9.	27	39	2.25	3.58	...	0.17
10.	42	35	2.13	...	2.95	0.61
11.	20	38	2.10	3.2	...	0.31
12.	28	38	2.27	...	3.5	0.27
13.	55	39	2.44	3.65	...	0.38
14.	24	37	2.05	...	3.0	0.57
15.	35	36	1.90	...	2.8	0.36
16.	31	37	2.07	...	2.9	0.68
17.	58	35	2.29	...	3.58	...
18.	36	39	2.48	...	1.0	...
19.	18	37	2.16	..	3.15	0.10
20.	26	37	2.32	...	3.5	0.18
21.	19	38	2.31	...	3.8	...
22.	55	37	2.24	...	3.18	0.61
23.	56	39	2.50	...	1.02	0.22
24.	57	30	3.31	...	3.9	...
25.	25	39	2.66	4.0	...	0.57
26.	43	37	2.29	3.7	...	...
27.	61	37	2.15	3.3	...	0.20
28.	24	38	2.65	4.2	...	0.13
29.	21	38	2.33	3.68	...	0.50
30.	22	39	2.59	4.0	...	...

No.	Age of the subject in years.	Volume of packed cells. %	mg. Phosphorus per 100 cc.			
			Whole blood.	Serum.	Plasma.	Cells.
Adult Females.						
31.	21	37	2.10	...	3.59	0.38
32.	39	39	2.50	3.7	...	0.63
33.	22	38	2.00	...	3.15	0.13
31.	22	30	2.60	...	3.15	1.32
35.	23	39	1.95	...	3.0	0.30
36.	25	39	2.18	3.45	...	0.20
37.	15	38	2.12	...	3.75	0.25
38.	15	39	2.55	4.25	...	...
39.	29	39	2.50	3.8	...	0.39
40.	48	38	2.48	3.95	...	0.09
Children.						
41.	5 M*	38	2.20	...	3.4	0.25
42.	3 F	39	2.48	...	4.1	...
43.	4 F	39	2.50	...	3.95	0.24
44.	9 M	39	2.60	4.0	...	0.41
45.	13 M	37	2.80	4.3	...	0.06
46.	4 M	38	2.55	...	4.0	0.09
47.	3 F	39	2.62	...	4.1	0.05
48.	2 F	39	2.70	...	4.3	0.20
49.	5 M	38	2.24	...	3.5	0.19
50.	8 F	39	2.43	4.0	...	...

* 'M' denotes Male, and 'F' Female.

The data presented in Table I shows that the inorganic phosphorus content of whole blood, with the exception of three cases, varies from 2.05 to 2.85 mg per 100 cc, while in the plasma the values range from 2.9 to 4.3 mg per 100 cc. The value for cellular inorganic phosphorus calculated from the factor varied from 0 to 0.64 mg, with the exception of only one rather high value of 1.32 mg. It is obvious that cells contain none or only very small amount of inorganic phosphorus.

The volume of packed cells varied more or less uniformly between 35 to 39 per cent. *Only in two cases a somewhat low value of 30 was obtained. Both* these cases showed low red blood cell count and low haemoglobin values while in all other cases these were found to be normal.

It appears that inorganic phosphorus content of the blood cells of children has a tendency to be lower than that of the adults. Behrendt (9) found as much as 5 mg of inorganic phosphorus per 100 cc. of the cells in children in contrast to the values found in this investigation which vary from 0 to 0.41 mg. per 100 cc of the cells. It has, however, not been possible to analyse the blood of infants of 2-3

months of age as was done by Behrendt. In general the results of this investigation confirm the findings of Brain, Kay and Marshall (1) ; Stearns and Warweg (2) ; Snyder and Katzenelbogen (4) that most of the inorganic phosphorus of the blood is present in the plasma and there is very little if any in the cells.

Pathological Cases :

The subjects selected for examination were in patients of the Medical Wards of the Mayo Hospital, Lahore. In the selection of patients, care was taken to ensure that as far as possible, a correct diagnosis was established after such clinical and laboratory examination which could be done under the hospital conditions. As the study was likely to be prolonged only such patients were selected which were likely to stay in hospital for at least a month. The blood was withdrawn from each patient weekly for examination.

Data showing the inorganic phosphorus content of the whole blood and plasma are presented in the following tables (II-VII).

TABLE II

Blood inorganic phosphorus in 4 cases of pyrexia of uncertain origin.

Case No.	mg. Phosphorus per 100 cc.			
	1st Day.	8th Day.	15th Day.	22nd Day.
(a) *Whole blood.*				
1.	2.10	1.92	2.21	2.05
2.	1.90	2.00	2.15	...
3.	2.20	1.85	2.03	2.10
4.	2.04	1.85	2.10	1.95
(b) *Blood plasma.*				
1.	3.22	3.35	3.15	3.05
2.	3.02	3.14	3.20	...
3.	3.40	3.00	3.17	3.08
4.	3.14	2.98	3.13	3.02

Observations recorded in the foregoing table show that the values for the inorganic phosphorus of the plasma, as well as those of the whole blood, were found to be within normal range. When the inorganic phosphorus content of the cells is calculated on the basis of the inorganic phosphorus of the whole blood minus the value of inorganic phosphorus in the plasma $\times \frac{60}{100}$, the values obtained are very small as compared to the corresponding values for the plasma.

TABLE III

Blood inorganic phosphorus in liver cases.

Case No.	mg. Phosphorus per 100 cc.			
	1st Day.	8th Day.	15th Day.	22nd Day.
Two cases of hepatitis.				
(a) *Whole blood.*				
1.	2.35	2.67	2.18	2.21
2.	2.52	2.41	2.60	2.40
(b) *Blood plasma.*				
1.	3.52	3.84	3.71	3.71
2.	3.63	3.75	3.01	3.28
Three cases of the cirrhosis of the liver.				
(a) *Whole blood.*				
1.	2.27	2.35	2.18	2.81
2.	2.05	2.15	2.00	2.45
3.	2.32	2.67	2.80	2.76
(b) *Blood plasma.*				
1.	3.20	3.45	3.45	3.68
2.	3.12	3.08	3.00	3.25
3.	3.55	3.41	3.50	3.31
Two cases of Cancer of the liver.				
(a) *Whole blood.*				
1.	2.18	2.32	2.40	2.45
2.	2.25	2.16	2.22	2.37
(b) *Blood plasma.*				
1.	3.52	3.65	3.50	3.60
2.	3.82	3.70	4.01	3.56
One case of atrophic cirrhosis of the liver.				
(a) *Whole blood.*				
1.	3.43	3.25	3.30	3.51
(b) *Blood plasma.*				
1.	4.20	4.15	4.08	4.32
One case of liver abscess.				
(a) *Whole blood.*				
1.	2.73	2.81	2.65	2.88
(b) *Blood plasma.*				
1.	4.05	4.20	3.95	4.00

Table III shows that the inorganic phosphorus in the whole blood and plasma of the cases described varies within normal range. In one case of atrophic cirrhosis of the liver the value for whole blood was repeatedly found to be higher than that in the blood of normal healthy people (Table I). This could be ascribed to two

causes. (i) It may be due to the reduction of the red blood cells, which commonly occurs in cases with the involvement of the liver. (ii) It is well known that in such cases blood serum phosphatase is elevated. With increased activity of the enzyme, the breakdown of the complex organic phosphate esters is increased resulting in an overall increase in inorganic phosphorus of the whole blood. To maintain the osmotic equilibrium between plasma and cells an increase of inorganic phosphorus in the cells is likely to occur and this increased inorganic phosphorus content of the plasma as well as of the cells leads to phosphaturea, which is not uncommonly observed in such conditions.

TABLE IV*

Blood inorganic phosphorus in cases of anaemia.

Case No.	mg. Phosphorus per 100 cc.			
	1st Day.	10th Day.	20th Day.	30th Day.
(a) *Whole blood.*				
1.	3.80	3.62	3.25	3.20
2.	2.86	2.95	2.70	2.61
3.	3.27	3.10	3.05	2.96
4.	3.63	3.60	3.35	3.22
5.	3.82†	3.64	3.50	3.42
6.	3.71‡	3.62	3.48	3.25
(b) *Blood plasma.*				
1.	4.38	4.22	4.28	4.31
2.	3.60	3.91	3.62	3.55
3.	4.15	4.10	4.02	3.90
4.	4.00	4.05	3.91	3.78
5.	4.20†	4.12	4.00	3.96
6.	4.05‡	3.90	3.83	3.85

Note.—

* Six cases of anæmia described here were studied over a period of six weeks. Liver extract therapy was continued during this time.

† This patient had at the time of admission to the hospital, an R.B.C. count of about 900,000 and volume of packed cells, 10 per cent.

‡ This patient had at the time of admission, an R.B.C. count of 600,000 and volume of packed cells 6 per cent.

Both patients Nos. 5 and 6 on admission were given blood transfusion, though neither of them benefitted by it.

The above results show an increase in the inorganic phosphorus content of whole blood. It was found that as the cell count of the blood decreased, the corresponding values for inorganic phosphorus in the whole blood rose. Again during treatment of the condition with liver extract as the R.B.C. count rose, the inorganic phosphorus content of the whole blood fell. This observation is interesting and can be explained on the phase theory. If the phase containing the

lesser amount of the solute diminishes to the corresponding advantage of the phase richer in the solute, then the percentage content of the whole with respect of the solute concentration, will rise. This is what seems to happen. When there is loss of cells, their place is taken by plasma.

TABLE V

Blood inorganic phosphorus of cases of bacillary dysentery.

Case No.	mg. Phosphorus per 100 cc.			
	1st Day.	8th Day.	15th Day.	22nd Day.
(a) *Whole blood.*				
1.	1.72	1.82	1.94	1.99
2.	1.58	1.50	1.61	1.65
3.	1.86	1.90	1.95	1.96
(b) *Blood plasma.*				
1.	3.10	3.15	3.09	3.20
2.	3.32	3.30	3.25	...
3.	3.60	3.41	3.40	3.52

Table V presents somewhat striking data. In these cases the values for the inorganic phosphorus of the blood plasma vary more or less within normal limits, but the figures for the inorganic phosphorus of the whole blood are unusually low. This may possibly be due to the dehydration of the body caused through repeated watery motions such as are common in bacillary dysentery.

TABLE VI

Blood inorganic phosphorus of cases of osteomalacia.

Case No.	mg. Phosphorus per 100 cc.			
	1st Day.	8th Day.	15th Day.	22nd Day.
(a) *Whole blood.*				
1.	1.90	1.85	1.93	1.81
2.	1.75	1.80	1.82	1.91
(b) *Blood plasma.*				
1.	2.65	2.80	2.94	3.00
2.	2.55	2.70	2.85	2.92

Table VI gives values for two cases of osteomalacia. The cases show typical deficiency of phosphorus as is evident from the values of blood plasma. The slow rise in the level of inorganic phosphorus in the whole blood and of the plasma during treatment is the result of vitamin D administration. The relative values for the

inorganic phosphorus of the whole blood and the plasma follow the same trend as in normal healthy subjects confirming our previous findings, that the greater part of the inorganic phosphorus is present in the plasma.

TABLE VII

Blood inorganic phosphorus of diabetic acidosis cases.

Case No.	mg. Phosphorus per 100 cc.			
	1st Day.	8th Day.	15th Day.	22nd Day.
(a) *Whole blood.*				
1.	1.13	1.18	1.25	1.40
2.	1.14	1.15	1.30	1.32
(b) *Blood plasma.*				
1.	2.20	2.30	2.40	2.51
2.	2.31	2.38	2.45	2.46

These two cases of diabetic acidosis showed the lowest of all values so far recorded for the inorganic phosphorus of the whole blood as well as the blood plasma. This may have resulted from glycosurea and the resultant diuresis, which drains away the inorganic phosphorus of the blood into the urine. These results also confirm the observation of Freudenberg (7) and Behrendt (9). The slow recovery resulted from constant insulin treatment.

CONCLUSIONS

The inorganic phosphorus content of the blood of 50 normal cases including adult males and females, and children of both sexes showed values ranging from 2.05 to 2.85 mg per 100 cc for whole blood ; 2.9 to 4.3 mg per 100 cc for plasma; and 0 to 0.64 mg per 100 cc for the red blood cells. It is thus obvious that the greater part of the inorganic phosphorus of the blood is contained in the blood plasma.

Some 26 pathological cases were similarly investigated including 4 cases of pyrexia, 9 of liver diseases, 6 of anaemia, 3 of bacillary dysentery, 2 of osteomalacia and 2 of diabetic acidosis. The results obtained confirmed observations made in the case of normal persons namely that most of the inorganic phosphorus of the blood is present in the blood plasma. The values in some cases were comparable with those of normal individuals but in some conditions the values were abnormally high as in cases of anaemia. In others they were found to be very much low as in cases of bacillary dysentery for whole blood and both for whole blood and plasma in cases of osteomalacia and diabetic acidosis. In anaemia cases it was somewhat striking to find that the inorganic phosphorus of blood changed inversely as the cell count. As the red blood cell count decreased, the inorganic phosphorus content of the blood rose. In osteomalacia the low values can be explained by the general depletion of the body in phosphorus.

It is well known that acids promote the hydrolysis of organic phosphate esters. Freudenberg (7) has observed in vitro experiments that acetic acid and even carbonic acid promote the breaking down of the organic phosphate esters. In the state of diabetic acidosis a large proportion of the organic phosphate esters appear to get decomposed by acid hydrolysis and are removed from the body by the attendant glycosurea and diuresis.

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THE ROLE OF PHOSPHORUS IN THE ANIMAL ORGANISM. PART II. ORGANIC PHOSPHATE ESTERS OF BLOOD IN HEALTH AND DISEASE

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The phosphorus content of blood in certain pathological conditions *e.g.*, rickets, *osteomalacia, tetany, hyperparathyroidism and other skeletal diseases* has been considered to be of clinical importance, but such studies have remained limited to the inorganic phosphorus content of the blood serum or plasma. In the performance of many functions of the body in which phosphorus is known to play a role, it operates chiefly through the medium of organic phosphate esters. Most of these organic phosphate esters have been found to be present in the blood cells and are associated with such functions as the maintenance of acid-base balance, buffer action or mineral reserve for the bones, and the metabolism of carbohydrates.

Striking changes in the various organic phosphate esters of the blood have been observed in animals with rickets by Rapoport and Guest (1). These authors have attached importance to the organic phosphate esters in the glycolytic cycle. Lohmann (2) observed that the Harden-Young and Neuberg esters as well as other phosphate esters were easily hydrolysable with hydrochloric acid after various intervals of time at the temperature of the boiling water. Such investigations have remained limited for a long time to the study of carbohydrate metabolism. Warweg and Stearns (3) later found that acid hydrolysis of organic phosphate esters from blood filtrates proceeded rapidly for ten minutes, became progressively slower until 12 hours, and then continued at a low but constant rate. Behrendt (4) utilising the method of Lohmann (2) of fractional hydrolysis of the blood filtrates noticed that the intracellular and extracellular organic phosphorus was almost equal.

This study was started with a view to investigate the relationship if any between intracellular and extracellular organic phosphorus in the normal organism and under

pathological conditions. If such a relationship existed, the variation from the normal, in the value of the various phosphate esters may throw light on the severity and the progress of recovery in the disease.

Analyses of samples of blood withdrawn from different persons among the members of the staff and student of the Punjab University Institute of Chemistry were made to determine the normal values, and 25 samples of blood from patients of the Mayo Hospitals, Lahore were examined to investigate the changes which may occur under pathological conditions.

To determine the amount of phosphorus existing in the blood in various organic ester combinations, Lohmann's method of fractional hydrolysis as modified by Behrendt was followed. Trichloracetic acid filtrate of the oxalated blood contains in addition to the inorganic phosphorus, the acid-soluble phosphorus of the whole blood. The acid-soluble phosphorus consists of adenosinetriphosphate, hexosediphosphate, hexosemonophosphate, and glycerophosphate. The preformed inorganic phosphorus could be directly estimated from the filtrate while esters could be hydrolysed to yield inorganic phosphorus which could then be estimated. Lohmann observed that these esters were hydrolysed during various intervals of time at 100°, namely within 7 minutes adenosinetriphosphate breaks down to give up its inorganic phosphate, and within 30 minutes phosphoric acid bound as hexosediphosphate was freed, while after 3 hours all the hexosemonophosphate released its phosphoric acid. Even at the end of 3 hours a certain portion of the organic phosphate remained unhydrolysed which consisted of glycerophosphates. This furnished a ready method of estimating the phosphorus in different ester combinations. Eight different fractions of phosphorus can be estimated:—

(*i*) Total phosphorus of blood.
(*ii*) Total acid-soluble phosphorus.
(*iii*) Preformed inorganic phosphorus.
(*iv*) Phosphorus present in combination with adenosinetriphosphate.
(*v*) Phosphorus present in combination with hexosediphosphate.
(*iv*) Phosphorus present in combination with hexosemonophosphate.
(*vii*) Phosphorus present in combination with glycerophosphate.
(*viii*) Phosphorus present in combination with blood lipids and as nucleic acid.

Technique.

22.5 cc. of a 10 per cent solution of trichloracetic acid was placed in a beaker and to this were added slowly while stirring 2.5 cc. of whole oxalated blood. The solution was allowed to stand in the ice chest for 5 minutes after which the protein precipitate was filtered through a 9 cm. Whatman No. 44 filter paper. Any other brand of an ashless filter paper would be equally suitable. Aliquot parts of this filtrate were used for the estimation of the various fractions of phosphorus in the blood.

6 cc. of the above filtrate was taken in a pyrex test tube marked 'A' and returned to the ice chest. This was used for the determination of preformed inorganic phosphorus.

To another set of three pyrex test tubes marked 'B', 'C', and 'D' were added 3 cc. of the filtrate into each, followed by 3 cc. of N/2 hydrochloric acid. The tubes were then tightly closed and placed into a boiling water bath. Tube 'B' was removed from the bath after 7 minutes, 'C' after 30 minutes, and 'D' after 3 hours and were placed into the ice chest immediately after removal.

Another 3 cc. of the trichloracetic acid filtrate were placed into a 100 cc. kjeldahl flask to which were added 5 cc. of 10-N sulphuric acid and 0.5 cc. of concentrated nitric acid. The contents were then heated over a naked flame until a clear liquid was obtained. Digestion could be completed if necessary by the addition of a few drops of perhydrol (30% hydrogen peroxide, Merck). The content of the flask were cooled under running water and transferred quantitatively to a 25 cc. measuring flask, rinsing the kjeldahl flask witt as little water as possible. To the measuring flask were then added 6 cc. of 5-N sodium hydroxide to neutralise partially the excess of sulphuric acid present. After cooling, the solution was made to the mark. 6 cc. of this solution was taken into a pyrex test tube marked 'E' and returned to the ice chest for the estimation of its phosphorus content.

Into another Kjeldahl flask 0.5 cc. oxalated whole blood was added and digested as explained in the last paragraph. The solution partially neutralised and made to 25 cc. 6 cc. of this solution was taken into another pyrex test tube marked 'F' and returned to the ice chest.

The inorganic phosphorus contents of the tubes 'A', 'B', 'C', 'D', 'E', and 'F' were then determined according to modified method of Bodansky (6) using 2 cc. of acid molybdate solution and 2 cc. of stannous chloride solution and allowing 30 minutes for the colours to develop completely before matching the colours with those obtained from standard phosphate solutions in a colorimeter. The amount of phosphorus can be read directly from Bodansky's tables in mg. per 100 cc. of whole blood for tube 'A', while for tubes 'B', 'C', and 'D', the values read were multiplied by 2 ; for 'E' the value read was multiplied by 25/3 ; and for 'F' it was multiplied by 5 to obtain values corresponding to mg. phosphorus per 100 cc. of whole blood.

If Po denotes the amount of preformed inorganic phosphorus, Px the amount of inorganic phosphorus after x minutes of hydrolysis, and Pa.s., the amount of total acid-soluble phosphorus, then the following will represent the various forms of inorganic and organic phosphorus in the blood.

Po	= Original or preformed inorganic phosphorus.
P_7-Po	= Phosphorus derived from adenosinetriphosphate.
P_{30}-P_7	= Phosphorus derived from hexosediphosphate.
P_{180}-P_{30}	= Phosphorus derived from hexosemonophosphate.
Pa.s-P_{180}	= Phosphorus derived from glycerophosphate.
Total P-Pa.s.	= Phosphorus derived from lipid, nucleic acid etc.

The value obtained for tube 'A' represents preformed inorganic phosphorus ; 'B' represents P_7, 'C' represents P_{30}, 'D' represents P_{180}, 'E' represents Pa.s. and 'F' total phosphorus. The phosphorus content in various ester combinations has been calculated in the manner explained above.

The results obtained for different fractions for the whole blood of 25 healthy individuals are shown in Table I.

TABLE I

Phosphorus content of Blood in Healthy Individuals.

No.	mg. inorganic phosphorus per 100 cc. of whole blood present as							
	Preformed inorganic phosphorus.	Adenosine-triphosphate.	Hexosediphosphate.	Hexosemonophosphate.	Glycerophosphate.	Acid soluble phosphorus.	Lipid and Nucleic Acid.	Total phosphorus.
1.	2.70	1.10	1.60	0.42	12.70	18.50	17.20	35.70
2.	2.20	2.30	1.50	0.80	13.20	20.00	11.80	31.80
3.	2.40	2.02	1.16	0.77	11.48	18.28	18.02	36.30
4.	2.00	2.82	1.22	1.54	...	...	...	43.20
5.	2.10	1.66	0.45	2.13	11.11	17.75	14.59	32.21
6.	...	2.70	1.05	0.75	...	21.70	15.55	38.25
7.	2.15	0.37	3.14	0.91	...	22.36	14.01	36.37
8.	2.51	0.29	1.46	0.90	...	19.80	11.14	33.91
9.	2.43	0.24	1.35	0.41	11.27	15.70	15.03	30.73
10.	1.93	2.04	1.33	1.03	...	...	...	...
11.	2.06	1.81	2.34	1.18	11.08	18.50	13.67	32.17
12.	2.05	0.96	2.01	2.01	...	22.80	8.05	37.85
13.	1.88	1.39	1.91	2.13	15.06	22.40	13.50	35.90
14.	1.92	0.71	1.10	1.52	12.05	17.30	...	...
15.	2.02	0.49	1.20	2.39	12.10	18.20	15.25	33.15
16.	1.75	1.65	0.89	2.35	11.56	18.20	14.40	32.60
17.	1.88	1.67	1.31	2.66	11.79	19.31	15.28	31.60
18.	2.07	1.91	0.52	3.60	12.95	21.05	11.77	35.82
19.	2.00	1.59	0.73	2.62	11.87	18.80	13.60	32.40
20.	1.92	1.40	0.68	1.91	11.42	17.36	15.12	32.48
21.	2.20	1.50	1.32	2.71	11.81	19.60	14.60	34.20
22.	2.16	1.20	0.90	1.95	10.81	17.01	15.69	32.70
23.	2.05	1.30	1.82	3.05	11.18	19.40	11.20	33.60
24.	1.98	2.00	1.50	2.10	10.65	18.23	13.85	32.08
25.	2.10	1.66	1.71	2.38	10.78	18.66	11.69	33.35

The range of variation as well as the mean value for each fraction in the different individuals is shown in Table II.

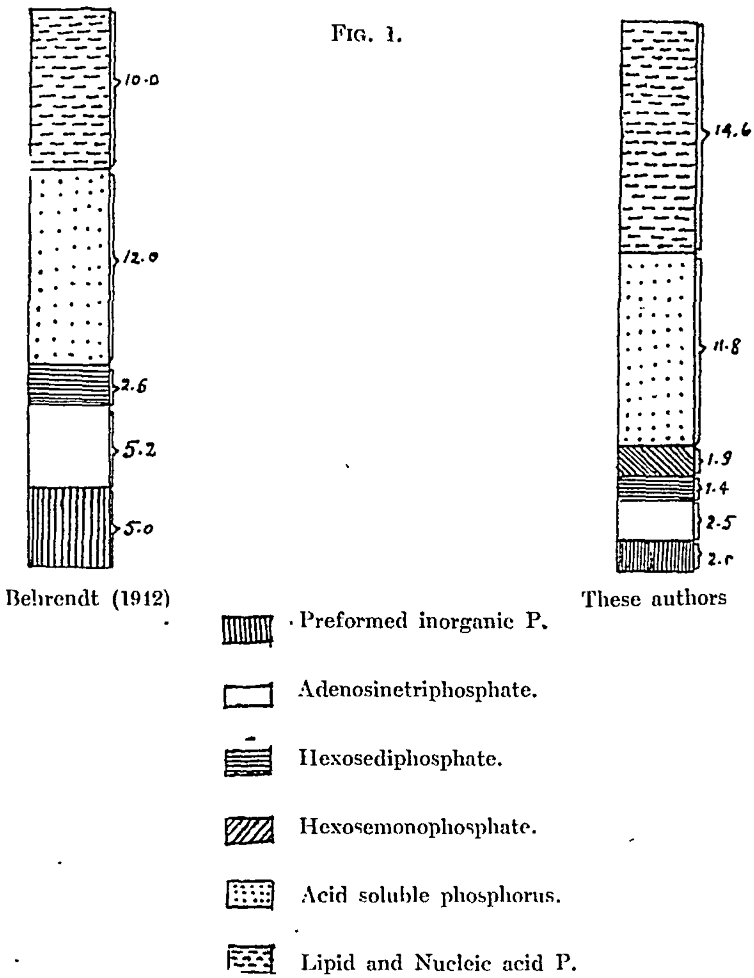
TABLE II

Average value of Blood Phosphorus Fractions in Healthy Subjects.

Name of Fraction.	mg. phosphorus per 100 cc. of the whole blood.	
	Range.	Mean.
1. Preformed inorganic phosphorus	1.88- 2.51	2.101
2. Adenosinetriphosphate	0.24- 2.82	1.472
3. Hexosediphosphate	0.45- 3.14	1.388
4 Hexosemonophosphate	0.44- 1.54	1.891
5. Acid soluble phosphorus	15.70-22.80	19.170
6. Glycerophosphate	10.65-15.06	11.852
7. Lipid and Nucleic acid	8.05-18.02	14.588
8. Total Phosphorus	30.73-43.20	34.928

Some of the values are also graphically shown in Fig. I. and for the sake of comparison values obtained by Behrendt (4) are also shown. The values for glycerophosphate are found to be the highest of all the different esters fractions occuring in the blood. In this connection it will be observed that the glycerophosphate is more stable than any other ester. It is possible that this can be formed from adenosinetriphosphate, hexosemonophosphate and hexosedisphosphate. The break-down of the glycerophosphate by the enzyme phosphatase occurs probably to a limited extent. Of the total acid soluble phosphorus, nearly two-thirds is present as the glycerophosphate fraction.

FIG. 1.



When these figures are compared with those obtained by Behrendt (4) a striking difference is observed. The preformed inorganic phosphorus of the whole blood obtained in our experiments is very much smaller than that found by Behrendt. In the same manner the three unstable fractions *viz.* adenosinetriphosphate, hexosediphosphate and hexosemonophosphate are also in much smaller proportion as in the case of preformed inorganic phosphorus.

Somewhat wide variations observed between the maximum and minimum values call for an explanation. The samples of blood were drawn at all times, without any regard to the interval between the food taken and time of the withdrawal of

the blood. This is bound to cause variations. It is well known for example that after a fatty meal the lipid content of the blood is considerably increased and therefore the samples of blood taken at such a time will give high values for lipid and nucleic acid phosphorus. With regard to the differences in the observations of Behrendt and ours, it may be pointed out that Behrendt selected only children for his study whereas the cases reported by us are all adults. It is also possible that climatic and dietary conditions in America which are different from those prevailing in India, may cause variations in the values.

The techniques followed by Behrendt (Fisk and Subbarow, 5) and by us (Bodansky, 6), are not likely to result in such wide variations as have been observed.

Recently extra and intracellular phosphorus determinations have been reported by Snyder and Katzenelbogen (7) who carried out the estimation by gravimetric analysis, using Hald's (8) blood ashing technique. They found that nearly all the inorganic phosphorus of the blood was present in the plasma. In two cases only they recorded values for inorganic phosphorus in the cells which they described as "traces" or "doubtful".

Cases of Anæmia.

The result of fractional analyses of phosphorus compounds in the blood of 16 cases of anæmia are shown in Table III.

TABLE III

Phosphorus content of Blood in cases of Anæmia.

No.	mg. inorganic phosphorus per 100 cc. of whole blood present as							
	Preformed inorganic phosphorus.	Adenosine-triphosphate.	Hexosediphos-phate.	Hexosemono-phosphate.	Glycero-phosphate.	Acid soluble phosphorus.	Lipid and Nucleic Acid.	Total phosphorus.
1.	2.89	0.18	1.56	1.26	14.89	20.83	10.37	31.20
2.	3.08	0.33	0.33	3.50	11.23	18.45	8.25	26.70
3.	2.86	0.55	0.54	2.91	10.39	17.25	8.85	26.10
4.	3.29	1.53	1.49	1.30	8.52	14.60	14.10	28.70
5.	3.27	0.50	0.89	1.64	10.53	16.83	9.92	26.75
6.	3.02	...	0.85	1.18	11.30	16.34	5.91	22.25
7.	3.09	0.07	1.70	1.16	9.82	15.84	7.86	23.70
8.	2.51	0.68	1.83	1.81	7.89	14.75	6.89	21.64
9.	3.22	...	0.68	3.40	9.03	16.33	10.27	27.60
10.	3.43	3.95†		1.68	11.84	20.90	6.00	26.90
11.	3.80	1.49†		2.51	10.70	18.50	7.80	26.30
12.	3.63	4.77*			9.76	18.16	...	...
13.	2.52	2.77*			8.83	14.42	4.08	18.50
14.	3.71	0.24*			7.21	11.16	8.64	19.80
15.	3.80	4.50*			3.82	12.12	12.68	25.80
16.	4.15	0.85*			5.21	10.21	8.79	19.00

† Represents total value for adenosinetriphosphate, and hexosediphosphate.

* Represents total value for adenosinetriphosphate, hexosediphosphate, and hexosemonophosphate.

The preformed inorganic phosphorus in the blood of these cases is of the same order as in the case of the blood of anæmia patients described in an earlier paper (Sehra and Ahmad, 9). The values for adenosinetriphosphate and hexosediphosphate phosphorus are much smaller than in normal individuals. These being easily hydrolysable seem to break down in this condition adding the released phosphorus to that of the plasma.

Total phosphorus of the blood has been considerably reduced in these cases. In some cases values as low as 18-19 mg. per 100 cc. were found. In different cases the total phosphorus content showed rather a wider variation. This was also found to occur in the value for glycerophosphate which showed a variation from 3.80 to 14.89 mg. per 100 cc.

Cases of Liver disease.

It is well known that in patients suffering from diseases with the involvement of the liver (Roberts, 10 ; Robinson, 11 ; Kay and Guyatts, 12 ; Sehra, Chopra and Mukerji, 13) the blood serum phosphatase level is elevated. It was therefore, considered pertinent to determine the level of blood plasma phosphatase. Table IV shows the result of analyses of the blood from seven pathological cases with the involvement of the liver.

TABLE IV

Phosphorus content of the Blood in cases of Diseases of the Liver.

No.	Plasma phosphatase.	mg. inorganic phosphorus per 100 cc. of whole blood present as							
		Preformed inorganic phosphorus.	Adenosine-triphosphate.	Hexosediphosphate.	Hexosenono-phosphate.	Glycero-phosphate.	Acid soluble phosphorus.	Lipid and Nucleic Acid phosphorus.	Total phosphorus.
Cirrhosis of the liver.									
1.	9.80	1.55	0.98	1.44	2.77	13.53	20.25	9.15	29.40
2.	10.20	1.99	0.37	1.21	1.87	14.29	19.66	15.04	34.70
3.	18.80	3.12	1.02	1.88	1.32	13.16	21.50	6.90	28.40
Cancer of the liver.									
4.	7.50	1.75	1.16	1.62	3.54	17.80	26.00	15.00	41.00
5.	8.00	1.92	...	1.21	1.81	11.07	16.01	...	...
Atrophic cirrhosis of the liver.									
6.	22.00	3.43	1.50	4.95	1.68	13.34	20.90	6.00	26.90
Liver Abscess.									
7.	7.00	2.73	0.07	1.04	2.63	10.65	17.75	15.59	33.70

The blood plasma phosphatase values were determined by the modified method of Bodansky (6). It is of interest to note that the glycerophosphate fraction has a tendency to be higher in these cases, the values for the blood plasma phosphatase being also higher.

CONCLUSION

In this paper the results of estimation of blood phosphorus present as preformed inorganic phosphorus, adenosinetriphosphate, hexosediphosphate, hexosemonophosphate, glycerophosphate, in combination with lipids and nucleic acid, total acid soluble phosphorus, and total phosphorus are described. The investigation covered 50 subjects including normal individuals, cases of anæmia and cases of liver disease.

The results of analyses showed that in normal individuals the preformed inorganic phosphorus of the whole blood varied from 1.88 to 2.54 mg. per 100 cc., while the pyrophosphate and hexosemonophosphate were found to vary from 0.24 to 2.82 and 0.14 to 4.54 mg. per 100 cc. respectively. The glycerophosphate fraction formed nearly two-third of the total acid soluble fraction. The average values for different fractions are found to be Preformed inorganic phosphorus, 2.104 ; Adenosinetriphosphate, 1.472 ; Hexosediphosphate, 1.388 ; Hexosemonophosphate, 1.894 ; Acid soluble phosphorus, 19.170 ; Glycerophosphate, 11.852 ; Lipid and Nucleic acid, 14.588 ; and Total phosphorus, 34.928.

In cases of anæmias the values for preformed inorganic phosphorus are higher than those observed in the case of normal individuals and are of the same order as were observed earlier in patients of anæmia by Sehra and Ahmad (13). The values for phosphorus found in the form of adenosinetriphosphate and hexosediphosphate are exceedingly low as compared to those found for the blood of normal individuals. Total phosphorus of the blood was also found to be considerably reduced in these cases.

In cases of liver disease no significant differences were observed in the values for various fractions, the value for the glycerophosphate fraction however, had a slight tendency to be higher and the total blood phosphorus somewhat lower. The values for plasma phosphatase were much higher than known for normal individuals.

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A PRELIMINARY NOTE ON THE COMPLEMENT-FIXATION REACTION IN KALA-AZAR WITH A SPECIFIC ANTIGEN AS AN AID TO DIAGNOSIS

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The early diagnosis of kala-azar has always been a problem of great importance. The aldehyde test which depends upon the increase of euglobulin in the serum, can be relied upon according to Napier (1) to give a definite and correct diagnosis of kala-azar of more than six months' standing. Chopra's antimony test though more delicate than the aldehyde test is not positive in every case of kala-azar and gives positive results in other diseases. The search for parasites by sternal or spleen puncture is not a very convenient method for routine diagnostic work. Besides it does not give positive results in all cases of kala-azar.

The diagnosis of Leishmaniasis by complement fixation reaction had been carried on with both specific and non-specific antigens. Earlier workers used liver extract both from human and canine kala-azar (2) and infected hamster's spleen (3) as their antigens. The preparation of specific antigens for complement fixation test from the culture of Leishmania was attempted by Auricchio (4), Ray J. C. (5), Zdrodowski and Woskressenski (6) and Da Cunha and Dias (7). Recently Niyogi and Ray (8) found positive complement fixation reaction in kala-azar cases using antigen prepared from flagellated culture of *Leishmania donovani.*

Greval, Sen Gupta and Napier (9) reported positive complement fixation reaction in kala-azar with Witebsky, Klingenstein and Kuhn antigen prepared from human tubercle bacilli. Though the reaction was of high order it was found positive in other diseases such as tuberculosis and leprosy and the physical basis of the reaction was stated to be a constituent of the serum which when sufficient in quantity also reacted with formaldehyde (9).

It appears that the tests mentioned above have not proved sufficiently specific and sensitive for early diagnosis of kala-azar. The present work was undertaken with a specific antigen prepared from culture of Leishmania donovani in order to find, if possible, a more specific test for the above purpose.

Preparation of Antigen :

Several recent strains of *Leishmania donovani* were cultured in Ray's media (10) at 21°C. An emulsion of 72 hours' growth of Leishmania was made with 0.25

per cent phenol in distilled water. This was matched with Brown's opacity tube No. 8 which was found to contain 80 million organisms per cc. The emulsion was kept in a glass-stoppered phial in an incubator at 37°C for 25 days. The phial containing the emulsion was shaken every day for 2 hours in a shaking machine having 120 oscillations per minute. At the end of this period the contents of the phial were centrifuged and the supernatant fluid was filtered through a filter paper. The filtrate was kept in cold and was used as antigen.

TECHNIQUE

In carrying out the complement fixation test for kala-azar, the method No. 4 of the British Medical Research Committee Report (1918) on the Wassermann test was adopted with certain modifications.

1. *Reagents :*

A. Antigen—its titration.

(i) *Anti-complementary power*

Different dilutions of antigen 1 in 5, 1 in 10, 1 in 20 and 1 in 40 were made and tested for anti-complementary activity against one minimum hæmolytic dose of complement as shown in Table I. The lowest dilution of the antigen in which complete lysis just occurred was 1 in 10. This was taken as antigen dilution for the test proper. The method of incubation of the mixture before adding the sensitised cells as given in the British Medical Research Committee report (1918) method No. 4 was modified as follows in order to obtain the optimum results. Instead of keeping ½ hour in room temperature and ½ hour at 37°C, the mixture was kept for ½ hour at 37°C and ½ hour at 15°C and for 5 minutes at room temperature.

TABLE I

Tubes.	Antigen.	Complement dil. (1 m.h.d. in 0.25 cc.)	Saline.		Sensitised cell Suspension.		
1.	0.25 cc. of 1 in 5	0.25 cc.	0.25 cc.	Mixed thoroughly and kept in incubator for ½ hour at 37°C, for ½ hour at 15°C and for 5 minutes at room temperature.	0.25 cc.	Left at 37°C for ½ hour	Readings then taken.
2.	0.25 cc. of 1 in 10	,,	,,		,,		
3.	0.25 cc. of 1 in 20	,,	,,		,,		
4.	0.25 cc. of 1 in 40	,,	,,		,,		

(ii) *Hæmolytic activity of the antigen :*

To ascertain that the antigen was not unduly hæmolytic 0.75 cc. of antigen in 1 in 10 dil. were incubated with 0.25 cc. of sensitised cells for ½ hour at 37°C. There was no trace of lysis showing that the antigen was not hæmolytic in that dilution even with three times the dose used for the test.

(iii) *Complement-fixing power of the antigen :*

For determination of the complement fixing power of the antigen high titre rabbit's immune serum against *Leishmania donovani* was used. The immune serum was prepared by giving 6 injections of living *Leishmania donovani* every fourth day into the rabbit's ear vein. The doses were gradually increased from 100 million to 1000 million organisms. Different dilutions of rabbit's immune serum were prepared. The complement fixing power of the antigen in 1 in 10 dilution against the serum was tested with 1½ m.h.d. of complement. The end-point was reached in a dilution of 1 in 6000 of the serum. This immune serum gave negative results in 1 in 10 dilution when tested with Wassermann antigen showing the high specificity of the antigen prepared.

In this connection it may be mentioned that different lots of antigen were prepared by other procedures such as extraction with alcohol, ether and acetone. All these were found to be lower in complement fixing power than the antigen used as determined against the rabbit's high titre immune serum. An antigen prepared with normal saline was found to be highly anti-complementary.

B. *Serum for test :*

The serum was inactivated at 55°C for ½ hour and was used in a dilution of 1 in 25 as a routine method. With higher dilutions of serum such as 1 in 50. 1 in 100 and 1 in 200 tests were carried out for determination of the strength of the complement fixing power. The complement added was kept steady at 1½ m.h.d. Sera from chronic kala-azar cases though they were positive even in 1 in 200 dilution gave doubtful results when dilutions of 1 in 5 were used. This was probably due to direct hæmolytic activity of serum in strong dilution.

C. *Hæmolytic system :*

Sheep's R.B.C. suspension was standardised according to the method described by Greval *et al* (11). This was so adjusted that seven drops (1 drop measuring 0·02 cc.) of 6 per cent suspension of sheep's corpuscles from a Wright's pipette of standard bore gave a direct reading of 51 on Sahli's hæmoglobinometer.

Sensitisation of R.B.C. suspension—This was made by adding standardised R.B.C. suspension to an equal volume of 10 m.h.d. of hæmolysin (1 m.h.d. of hæmolysin giving a titre of not less than 1 in 1000). This was then kept in incubator at 37°C for half-an-hour and at 15°C for another half-an-hour.

Complement—Minimal hæmolytic dose of complement was determined without using the antigen. Different dilutions of the complement (pooled guineapig sera) were prepared. To 0.25 cc. of each dilution was added 0.5 cc. of saline and the mixture was incubated, as mentioned before at 37°C for ½ hour, 15°C for ½ hour and finally for 5 minutes at room temperature. 0.25 cc. of sensitised cells was then

added to each tube and incubated at 37°C for $\frac{1}{2}$ hour before the results were taken. The highest dilution of the complement in which complete lysis occurred was taken as 1 m.h.d. of complement.

II. *The Test :*

In carrying out the test $1\frac{1}{2}$ m.h.d. of complement were used for serum control, antigen control and the test proper. Another antigen control was set up with 1 m.h.d. of complement in which lysis should be just complete to show that the anti-complementary activity of the antigen was 1 m.h.d. of complement.

The method is shown in the following table.

A. *An ensemble*

TABLE II

Tubes.	Serum dil. 1:25	Complement dil.	Antigen dil. 1 in 10	Saline.		Sensitised cells.				
1. Serum control.	0.25 cc.	0.25 cc. ($1\frac{1}{2}$ m.h.d.)	...	0.25 cc.	Thoroughly mixed, left at 37°C for $\frac{1}{2}$ hour, for $\frac{1}{2}$ hour at 15°C and for 5 minutes at room temperature.	0.25 cc.	Incubated at 37°C for $\frac{1}{2}$ hour.	First reading of the results taken.	Left overnight at 15°C.	Second reading of results taken.
2. Test proper	,,	,,	0.25 cc.	...		,,				
3. Antigen control with $1\frac{1}{2}$ m.h.d. in 0.25 cc. of complement dilution	...	,,	,,	0.25 cc.		,,				
4. Antigen control with 1 m.h.d. in 0.25 cc. of complement dilution	...	0.25 cc. (1 m.h.d.)	,,	,,		,,				

B. *Reading of the results :*

(*i*) Complete inhibition of lysis = +
The supernatant fluid was absolutely colourless on next day.

(*ii*) Trace of lysis = T
The supernatant fluid showed a slight tinge of colour on the next day.

(*iii*) More than a trace of lysis = ±. The supernatant fluid was coloured but a slight deposit of cells still remained.

(*iv*) Complete lysis = –
+ and T were regarded as positive, ± as doubtful and – as negative.

For differentiating between +, T and ± the tubes were kept overnight at 15°C and readings were taken on the next day when the differences between them were brought out better.

C. *Results are given in the following table.*

TABLE III

Diseases.		No. of cases.	Complement fixation in serum dilution of 1 in 25	1 in 50	1 in 100	1 in 200
Kala-azar— 30 cases	A. Aldehyde test positive	17	+	not done		
	B. Aldehyde test negative, diagnosed on clinical grounds and response to antimony treatment	8	+	+	T	–
		3	+	+	+	T
		2	+	±	–	–
Non-Kala-azar— 46 cases	Including Typhoid, Malaria, Tuberculosis, Leprosy, splenic anæmia, Cirrhosis of liver, leukæmia, catarrhal jaundice, liver abscess, severe anæmia, Heart-failure, nephritis, peritonitis, pleurisy and pregnancy.	46	–	–	–	–

Remarks

The complement fixation reaction with the antigen used is highly specific for kala-azar and the fact that positive reaction was obtained by this test in cases where the aldehyde test was negative would indicate that early diagnosis is possible by this method. Further investigations are required to see whether the test is positive in other forms of Leishmaniasis as well. In the series of non-kala-azar cases various disease group including leprosy and tuberculosis have been excluded even in dilution of the serum lower than 1 in 25. The reaction is highly sensitive. Because of the sensitivity and specificity of the reaction both + and T were grouped as positive and ± as doubtful in the interpretation of the results.

Summary

1. A specific antigen for complement fixation reaction in the diagnosis of kala-azar, was prepared from flagellated cultures of *Leishmania donovani* in Ray's media.

2. The technique of complement fixation reaction followed was according to the No. 4 method of the British Medical Research Committee report (1918) on Wassermann reaction with certain modifications.

3. In 30 cases of kala-azar studied, positive results were obtained in all the cases. Negative results were obtained in cases of other diseases.

4. Sera from cases of kala-azar were generally anticomplementary and had direct hæmolytic activity in strong dilutions. So 1 in 25 dilution of serum was used for the test to exclude these two possibilities.

5. To find out the end point of the reaction the dilution of the serum was gradually increased keeping the complement fixed at $1\frac{1}{2}$ m.h.d.

CONCLUSION

The complement fixation reaction described in this paper appears to be highly specific and sensitive and of great diagnostic value for kala-azar. The test becomes positive in early cases of kala-azar when the usual serum tests are negative. It can be conveniently employed as a routine test for the early diagnosis of kala-azar.

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FOOD*

I am deeply sensible of the honour you have done me by asking me to preside over the Chemistry Section of the Indian Science Congress this year. I am conscious that there are far more qualified individuals who could have discharged the presidential responsibility with much greater competence. I have, however, humbly accepted this office taking it as a mark of recognition of the importance of biochemistry, food and nutrition in the world of science.

I hope I shall be pardoned if I say that biochemistry has been a Cinderella of the sciences in India and inspite of the fact that the science of food and nutrition is probably the most important science concerned with the well-being of the people, its recognition so far has been relatively very meagre. Long before this war, all these subjects began to be actively pursued in Europe and America and the war brought into bold relief the importance of these sciences and of their application throughout the world. That food is the first essential of all living things is a truism. But the extent to which food, qualitatively and quantitatively inadequate, has affected the health of vast masses of the population began to be realized barely 15 years ago. Food and nutrition surveys in Britain and America revealed that about half the people of these rich countries were not at an optimum level of nutrition according to modern standards. Although precise figures are not available for India, it would not be a wild guess to say that probably 80 to 90 per cent of her people are either under-nourished or mal-nourished or both. That we are perpetually walking on the brink of the precipice was shown when we fell over the precipice in the tragic year of 1943. A social, political and economic order, which has to watch on when millions die of starvation, has obviously long outlived its usefulness and become a clog in the wheel of progress.

In India before the war scientific as well as public recognition of the basic importance of food was exceedingly inadequate. In the vast majority of our universities and scientific institutions, including medical, there is still no full-fledged department of biochemistry and the volume of work on food and nutrition produced in India is relatively very small compared with that in America, Russia and England. I would make here a special appeal for the organisation of departments for these studies in all the universities and medical institutions in India and would also urge the creation of special institutions devoted to particular subjects like biochemistry, nutrition and food-technology.

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* Presidential address by Dr. B. C. Guha, Ph.D., D.Sc., F.N.I. to the section of Chemistry, Indian Science Congress, delivered on 3rd January, 1916 at Bangalore.

In view of the importance of the science of food I make no apology for taking this subject for a brief discussion today.

The development of science has to be envisaged in both pure and applied aspects. To pursue exclusively the applied side ignoring fundamental scientific studies would be, as J. J. Thomson once said, like ploughing a field and forgetting to sow the seeds. It is true that in this country even what we know about the science of food and nutrition has not been applied to any appreciable degree and this is a matter which requires the first attention of the Government and the public. But it is also true that progress on the applied side, if it is to be maintained on right lines, requires that the path should be continuously illuminated by the light of pure research. As an example it may be pointed out that in the last century we were thinking mainly of the major food constituents—proteins, fats and carbohydrates—in connection with human nutrition, now as a result of the discovery of vitamins the applied aspects of nutrition have been to a large extent revolutionized. In the last two or three decades the recognition, identification and production of vitamins have proceeded at an unprecedented speed and these discoveries have proved to be of inestimable value during the war-time conditions of food supply. Under Lend-Lease enormous quantities of synthetic ascorbic acid have been sent by America to other countries of the world where the sources of vitamin C were limited. The production of synthetic vitamins of the B group has led to the fortification of 80 to 90 per cent. of the total bread produced in America. I shall refer to this subject later again. I am pointing this out in order to indicate that the fountain of fundamental researches must be maintained if the field of applied nutrition is to be kept continuously irrigated.

Fundamental Studies

During recent years quite a large number of vitamins of the B-group have been recognised and synthesised. We have now synthetic thiamin, riboflavin, niacin, pyridoxin, pantothenic acid, biotin and *p*-aminobenzoic acid. Folic acid is considered a new vitamin which may be identical with the grass factor, which was indicated by the writer in a paper in 1931. Vitamins A and A_2 and different D vitamins have been recognized. Vitamin C has been synthesised. A new vitamin P has been recognized. Vitamin E has been synthesised. The vitamin-like nature of certain unsaturated fatty acids is now largely accepted. There is some evidence for the existence of new factors, vitamins B_{10} and B_{11} (Elvehjem). There is also evidence of growth-promoting activity for chicks in fish-pressed water which is probably not accounted for by known vitamins. Thus the discovery of still more new vitamins is in the offing.

Our progress in the knowledge of the fundamental scientific nature of vitamins and particularly their synthesis, which has made some of them readily available, have greatly stimulated our understanding of their mode of action. We are now aware that a number of the B vitamins are concerned in the enzyme systems responsible for biological oxidation. The stages at which the vitamins participate in the metabolic process are a little better understood than before in one or two cases. The results obtained by S. Banerjee indicate that vitamin C may be connected with insulin secretion which would probably be the first indication of the relation between a vitamin and a hormone. The observations of S. Roy in our laboratory

indicate that the bio-synthesis of vitamin C in rats involves thiamin and probably also riboflavin. Some of the B vitamins and vitamin C would thus appear to be biochemically related. That the path of the bio-synthesis of vitamin C, which is stimulated under the influence of narcotics as was shown by King and his co-workers, constitutes probably a diversion of the normal path of carbohydrate metabolism, is indicated by the work of B. Ghosh, S. Roy and S. K. Roy. That vitamin D is concerned in calcium and phosphorus metabolism is well known, but the details of the process are not yet completely clarified. The stimulating effect of vitamin E on the utilisation of vitamin A has recently been shown by Harris at Rochester, N. Y. The lack of vitamin E has been shown not only to produce sterility but also to produce muscular dystrophy. It has been shown that partial deficiency in vitamin E is more widespread than has been suspected. Work has been carried out in Prof. Elvehjem's laboratory on the synthesis of certain vitamins of the B group by intestinal micro-organisms. It would appear that this might be an appreciable source of these vitamins to man which would supplement the ingested vitamins. This again is worthy of further investigation. Substances which are called anti-vitamins have been recognised and it is interesting to see how desthiobiotin is an antagonist to biotin. Similarly, riboflavin is antagonised by *iso*riboflavin which contains two methyl groups in 5, 6 positions instead of 6, 7 positions as in riboflavin. Thiamin is antagonised by pyrithiamin and nicotinic acid by β-acetyl pyridine. The explanation suggested for this highly interesting phenomenon is that the anti-vitamins compete with the vitamins in relation to the enzyme systems involved in cellular respiration. However, the claim by Woolley that glucoascorbic acid is an anti-vitamin appears to have been disproved by S. Banerjee. How slight changes in the structure of the vitamins convert them into anti-vitamins is indeed a field for interesting investigation. The relation of certain vitamins like *p*-aminobenzoic acid to bacterial enzyme systems and the competition of sulphonamide drugs with it, which appears to be largely responsible for the anti-bacterial action of sulphonamide drugs, has indicated new lines of fruitful study. The above illustrations indicate far too briefly the new lines of fundamental vitamin studies, which besides extending our bounds of knowledge, will have important repercussions in the field of applied nutrition. It is important to recall that even 20 years back we were completely ignorant of the mode of the biochemical action of vitamins and now we may at least claim to have the glimmering of light on that highly complex question. While I have the feeling that further light will reveal still greater complexity of the problem, yet it is no mean satisfaction to reveal a complexity instead of being ignorant of it. Doubtless, with the present tempo of progress, complexity will both be revealed and unravelled in which I would urge Indian workers to take part. As I have said, the implications of these researches in the applied field are also very far-reaching.

Striking developments have taken place also in other directions. The phenomenon of transmethylation in the tissues and the importance of labile methyl groups in certain dietary constituents have been recognised by du Vigneaud and co-workers. Light has been thrown on enzyme systems concerned in the metabolism of amino-acids by N. Das, O. Warburg, F. Kœgl and others. The phenomenon of transamination has been discovered largely as a result of the work of Braunstein. The dynamics of amino-acid metabolism have been studied by the use of heavy nitrogen and heavy hydrogen for labelling the molecules (du Vigneaud, Schoenheimer). The

theory of a dynamic equilibrium between tissue and plasma protein seems now to be well established by the use of the same isotope tracer technique (Whipple). Researches have been carried out on the metabolism of fats in a similar way by the use of deuterium (R. S. Harris). Similarly, by the use of radioactive isotopes, calcium, phosphorus and iron metabolism has been sought to be more closely studied and mineral metabolism generally has received a good deal of attention including that of trace elements. With radioactive iron the very interesting and useful observation has been made that while human beings absorb ferrous iron preferentially, a marked preference of ferrous over ferric iron was not observed in the case of dogs, indicating a definite species variation in biological reaction. Carbohydrate metabolism still continues to be a subject for fruitful studies because of its varied connection with proteins, fats, vitamins, hormones and enzymes.

Work has continued to be carried out on the important question of the human requirements of different nutritive factors, including vitamins. Mitchell makes the generalisation "that the body's primary need for nutrient is for food energy and that its need for most of the specific essential nutrients is in proportion to the amount of food energy consumed." The minimum requirement of protein appears to be also related to basal metabolism (Terroine-Smuts relationship). However, our knowledge about the requirements of nutritive factors is still incomplete and much further work needs to be done on this subject as recent researches indicate that the requirements of various factors are influenced by the nature and quantity of other constituents of the diets. A striking example is the sparing action of fat on thiamin as was observed by Lepkovsky and the differential sparing action of different fats and oils as was observed by the writer many years ago. The deleterious effect of feeding an excess of certain vitamins has also been established including the toxic effect of a large consumption of vitamin A. The poisoning of Arctic explorers by the consumption of the liver of polar bears has been traced to its large content of vitamin A.

Interesting observations have recently been made on the alteration of amino-acid metabolism in moulds and also of their ability to synthesise vitamins by exposure to x-rays. It would appear that the use of radiations of different kinds would in future years throw more light on biochemical transformations in protoplasm. This should be a line for fruitful study. May I hope that some day we shall be able to interpret living phenomena in terms of energy levels and the newer developments in atomic physics.

A passing reference may also be made to the medical implications of some recent nutritional researches. The use of certain vitamins for the treatment of various deficiency diseases is quite well-known. The new vitamin folic acid has been claimed to be effective in the treatment of pernicious anaemia. Riboflavin has recently been recommended for the treatment of the condition of perleche of which however, full confirmation is necessary. The therapeutic effect of glutamic acid on the treatment of the petit mal type of epilepsy has been claimed, while it has been found quite ineffective in the treatment of grand mal. Highly interesting have been the observations of Zimmerman and Ross who claim that incorporation of *l*-glutamic acid in the diet increases the learning ability of rats. It reduces the time required to learn a maze to a 3rd or a 4th of the time needed by the control animals.

Glycine as a contrast had no effect. These results are curious considering that the diet normally consumed contains glutamic acid in the protein. However, these observations would be interesting to our young learners, and if glutamic acid can help them before an examination, it will be a tribute to biochemistry!

Food Technology

The need of pursuing fundamental researches on biochemistry and nutrition has been indicated above. Another line in which our country is far too deficient is the applied science of food technology including the processing of foodstuffs in order to preserve or enrich them. The production of food in our country is not adequate if we are to plan nutrition for the people according to the standard prescribed by the National Research Council of America or even at a lower standard. Secondly, a considerable part of our food-stuffs perishes annually because of rodents and insects which abound in tropical countries. It has been roughly estimated that about three million tons of food-grains alone are lost in India owing to damage by rodents, insects and moisture. If we consider all food-stuffs in this country, including such perishable materials as fish, milk, meat, vegetables etc., the extent of our loss may be guessed. There is not much value in a "Grow More Food Campaign" if a considerable part of the food produced perishes for lack of development of food technology in this country.

Regarding grains the main question is that of drying the grains to a low moisture content, preferably of the order of 8% which makes the grains resistant to insects and moulds. This drying is normally carried out in this country in the sun, which, while it is rather inexpensive, has got its disadvantages because of the fickleness of weather, dust etc. In the West grain-dryers are extensively used for rapid and uniform reduction of the moisture content of grain. Once dried, the grain has to be stored in proper bins or warehouses so that re-absorption of moisture may be as little as possibe. It is obviously no use drying the grain if it is stored later in a condition facilitating moisture re-absorption. Fumigation is resorted to frequently and a number of fumigants have been used, among them more particularly ethylene oxide and ethylene dichloride in America. Various inert dusts have been used also to prevent insect infestations. In recent months experiments have been carried out with D.D.T. and 666 with good results. All these researches have to be actively pursued as they have a tremendous bearing on the problem of the preservation of the food grains in this country. The deterioration of food-grains, particularly during the war years, has been most distressing and enormous quantities of such food grains have been thereby lost to a people already suffering from want of food. Prof. V. Subrahmanyan has made commendable efforts to recondition deteriorated food-grains.

In Britain in course of the war a national flour was introduced which was 85% extraction flour so that more of the B vitamins and iron might be retained. Some time back the extraction was reduced to 80 per cent. To this flour was added about 0.16% of calcium carbonate so as to fortify the flour with calcium in order to counteract the anti-calcifying effect of phytic acid contained in the flour.

In the U.S.A. about 80 to 90 per cent of the bread is being enriched with thiamin, riboflavin, nicotinic acid and iron and the extra cost, as Dr. R. R. Williams informed me, came to barely -/8/- annas per head per year.

A process of "converting" rice has been developed by Huzenlaub by which the vitamins are driven towards the interior of the grains so that during milling and polishing the rice grain does not lose much of its vitamin B factors. The method makes the rice harder and, therefore, more resistant to insects. There is also less loss due to powdering during the milling process. However, it is not yet clear whether this method gives an outstanding advantage over the parboiling process which also helps the vitamins to be retained in the rice grain during the milling process and of which Huzenlaub's process is an improved and mechanised modification.

A method has been developed for fortifying rice with the B vitamins and then coating the rice grain in such a manner that during the washing of the rice the added vitamins are not leached away. This fortified rice which is called a premix may then be mixed with a very large bulk of unfortified rice in order to give a rice of high average vitamin content. All these methods are worthy of consideration in connection with the development of food technology in this country so that our people may be more adequately fed quantitatively and qualitatively.

It may be stated in passing that the Ministry of Food specialists have found zinc phosphide to be the most effective agent for destroying rodents.

Among the different methods of food technology, canning, dehydration, freezing, etc., the freezing technique has made the most headway in America in recent years. It is high time that in India refrigeration were extensively established for preserving food-stuffs which undergo spoilage by attacks of insects, moulds and fungi as well as by autolysis at a higher rate in a tropical climate than in the temperate climate of the West. The Birdseye quick-freeze technique has been developed greatly in the United States. The principle of the method consists in cooling down the food-stuffs very quickly to a temperature of the order of—30°F. and then storing the frozen product somewhat below 0°F. until the time of consumption. In actual practice the factories are located where the raw materials are available. The factories quick-freeze the fresh food-stuffs and then put them in warehouses maintained at a temperature of 0°F. to—18°F. From there they are sent in insulated transport, which is cooled inside by some refrigerant, and brought to the consumption centre where they are again stored in warehouses at temperatures of 0°F or below. The retailers obtain their daily supply from these warehouses which they keep in their ordinary refrigerators and sell during the day. The development of refrigeration process for food-stuffs however, requires a fairly extensive organization. Once this organization is achieved, the process is simple. The advantage of quick-freezing is that it is the only process whereby the food-stuffs are retained in a condition which is practically indistinguishable in taste and flavour from the fresh food-stuffs. We have tasted haddock which was preserved for three years in such a manner that it was not possible to tell it from a fresh haddock when cooked and tasted. On the other hand refrigeration of food-stuffs does not save space as dehydration does. But canning as an alternative procedure takes even more space. There are several methods by which this quick-freezing technique has been applied.

(1) *The Birdseye Process :*

In this process the Birdseye multiplate freezer is used which consists of a number of super-imposed hollow aluminium plates through which the refrigerant is running.

These plates are hydraulically operated so that the distance between them can be adjusted. The food-stuffs are wrapped in waxed paper, put in a card-board carton and are placed between two hollow plates and then the plates are adjusted so that they are brought just in touch with the carton. Freezing takes less than two hours after which the food package can be removed and stored in the cold warehouse. In recent years mobile units of this type have been built which, complete with everything, can be moved from place to place if production requirements make that necessary. This is a process which is extensively used in many fish factories in America. For freezing vegetables it is usual to blanch them before quick-freezing to destroy the enzymes.

(2) *Blast Freezer :*

In this system a cold blast of air cools down the food-stuffs. The blast is allowed to go through a chamber containing horizontal coils with fins to facilitate cooling. The refrigerant is allowed to flow through the coils and the food-stuffs in trays are placed on the coils. Alternatively the food-stuffs in trays can be placed on trucks which are moved into a tunnel through which a cold blast is passed.

(3) *Finnegan Tubes :*

These tubes are used particularly for the freezing of fruit juices like orange juice. In America ordinarily orange juice is canned or bottled with or without previous concentration. But during recent years the method of freezing by Finnegan Tubes has been adopted. The cans containing the deaerated orange juice and closed under vacuum are placed in inclined tubes through which refrigerated alcohol at approximately—35°F. flows at a high velocity. As the cans pass through the Finnegan Tubes under gravity, they come in contact with the cold alcohol and the contents of the can freeze very quickly. The frozen juice is considerably more akin to fresh juice in taste and flavour on thawing than the ordinary canned or bottled orange juice of commerce.

There are many variations of the above methods. The workers of the Massachusetts Institute of Technology had kept completely cooked meals in a frozen condition at —10°F. for a pretty long time. This could be warmed up at the time of serving and had not deteriorated appreciably in taste and flavour.

It should be stated that quick-freezing has been found to be a first class method for preserving fish and vegetables. Many fruits also can be quick-frozen, particularly, straw-berries, goose-berries, cherries etc. But many other fruits would not keep well in a quickfrozen condition. For such fruits gas storage, that is to say, storage in an atmosphere containing a certain ratio of carbon dioxide to air has been found to be very effective. Under these conditions the respiration of the fruits is retarded but not completely stopped. Gas-stored fruits are maintained at a temperature at or slightly above the freezing temperature. Apples and pears behave well to gas storage and it is probable that mangoes also would do well but the exact conditions would have to be worked out.

Canning is an established process and need not be referred to in detail. In recent years they have developed many automatic machineries for the canning industry including continuous pressure cookers and pressure coolers.

Dehydration has particularly come into prominence during the war as it is meant both for the preservation of food-stuffs and for reducing their volume and weight. Dehydration of fish, for instance, would reduce the actual consumable part of the fish to only about 1/20th of its original weight. This means considerable saving in space which is so important in war-time. Dehydration of vegetables has been quite extensively carried out during the present war in this country and also elsewhere. The method consists in putting the washed, sliced and blanched (usually in sulphite solution) vegetables on trays which are placed in trucks, which are led through tunnels under a blast of hot air. There are tunnel systems of various designs in different countries, sometimes using a single counter-current air blast, some times a parallel flow air blast and often a combination of the two. The last is the most effective. There are methods for compressing some types of dehydrated vegetables whereby the space occupied can be further reduced. Dehydrated food-stuffs are preferably packed in tins in an atmosphere of nitrogen or carbon dioxide. The storage quality of dehydrated vegetables is obviously of great importance and quite extensive studies have been carried out on the changes which occur in dehydrated vegetables during storage. If there is air there is deterioration. There is also a deterioration from the Maillard reaction between reducing sugars and amino-acids. The browning thus caused is non-enzymatic. It is usual in dehydration practice to blanch the vegetable carefully so that the enzymes which might cause subsequent enzymatic deterioration are pretty effectively destroyed. Pre-cooked dehydrated vegetables have also been prepared. They reconstitute in water considerably more quickly then ordinary dehydrated vegetables.

Dehydration of fruits is an established industry in the U.S.A. In this country in the N.W.F.P. during the present war, fruits like peaches, apricots, plums, pears, etc. have been sulphured, sun-dried, washed with water and sulphured again. They have been subsequently dehydrated in a tunnel dryer. Straight dehydration of the fruits has also been practised recently both in India and in the U.S.A. in which the fresh fruits are blanched, sulphured and dried in a tunnel dryer like vegetables. This gives a good product of fairly uniform quality if the conditions of processing are carefully observed. Experiments are now in progress in the N. W. F. P. to see if dipping in metabisulphite solution may replace sulphuring.

Temperature has an important effect on the rate of deterioration during storage of dehydrated vegetables and fruits. Therefore, it is desirable that all dehydrated food-stuffs should be kept in a cool place if a fairly long storage life is desired. Dried fruits, in fact, deteriorate 2.5 times faster for every 10°F. rise in temperature.

Meat has been dehydrated during this war in India, Australia and Argentina. The method of producing sliced dehydrated goat's meat in India is more or less similar to that of dehydrating vegetables. Precooked minced meat has been produced in Argentina and Australia.

Precooked minced fish has also been dehydrated on a pilot plant scale which gives a quite palatable product in the form of fried fish cakes. I wonder whether the shark flesh, which is available in large quantities in the shark liver oil industry in India, may not be dehydrated to produce a good protein food. Shark flesh contains a little urea which unfortunately gives a slight after-taste of ammonia, but possibly the urea may be leached out from shark flesh before dehydration.

New methods of dehydration have been tried. Fruits, for instance, have been dehydrated in America under vacuum at an average temperature of 120°F. to give fairly good products. This has the advantage over tunnel drying in that it gives a quicker turnover and also there is less heat damage to the fruits.

Another method is to dry food-stuffs in the frozen condition as plasma is dried. This has not yet come into industrial practice because the economics of the process so far are against it. But eggs, for instance, which are normally spray-dried give a first-class product if they are dried under high vacuum in a frozen condition. The flavour is almost like that of fresh eggs, which is hardly the case with spray-dried egg powder. Attempts are being made to freeze-dry milk also which is normally spray-dried or roller-dried. Freeze-dried milk is considerably better in flavour and nutritive quality than the other forms of dried milk powder. This method of drying seems to be a promising line of development particularly for milk and eggs. I have seen freeze-dried straw-berries, peas etc., which are also good but so far the cost of freeze-dried vegetables has been considered to be too great. It may not, however, always be so.

Another new method of drying has been by heating in oil under vacuum. Potatoes, fish, etc. can be dried quickly under vacuum if they are heated in oil. This is a method also capable of development in India. The product, however, contains always a certain quantity of oil, which probably would not be unacceptable for fish and vegetables.

Radio-frequency energy has also been used for dehydration but so far not with industrial success.

For drying of fish, dry-salting is an important process which is fairly extensively practised in Europe and America and it is probable that our indigenous method of fish drying may be considerably improved in the light of the experience of the dry-salting industry. Among all the processes of preservation of fish, dry-salting would be the cheapest. But it would not obviously give as good a product as other methods of preservation, particularly refrigeration.

Smoking of fish is also a method of preservation of fish but smoked fish will have to be popularised in India if this method has to be developed.

Dehydration work has gone on so far in America that one could get a complete meal from dehydrated food-stuffs including dehydrated icecream and dehydrated chutneys.

While considering alternative processes of preservation, canning, refrigeration, dehydration, etc., it is important to remember that they should not be regarded to be so much competitive as co-operative as there are circumstances under which one or other process would be suitable and some times the same factory may carry out all the three processes even with the same food-stuffs. The development of food-technology in this country on right lines would require considerable planning.

Under food-technology we may also refer to the production of vitamins and food yeast in this country. Enormous quantities of all the important vitamins are now being produced particularly in the U.S.A. and it is of great importance that for a country like India with a vast mal-nourished population we should produce them also in required quantities. The synthetic vitamin industry, however, is bound

up with a basic chemical industry and solvents industry. But it is time that the development of this industry is considered in detail in connection with the development of related industries so that the production might be economic.

The method of production of food yeast has been developed by Dr. Thaysen and this method has been found to give a good yield of food yeast of high nutritive value to the extent of 25-30% of the molasses used. Since over four hundred thousand tons of cane molasses are produced in India annually the production of food yeast from a part of this molasses is worthy of consideration.

In connection with food-processing industries it should be mentioned that the development of this industry not only preserves large quantities of perishable food-stuffs but it also helps to take off the seasonal gluts, thus making the price even throughout the year. At present due to seasonal gluts in, let us say, mangoes, lichies or hilsa fish, the prices are often uneconomic to the producer while considerable quantities perish because they cannot be sufficiently quickly transported to consumption centres. At the same time, in off seasons these food-stuffs are either unavailable or available at a very high price. An even price throughout the year would be good both to the consumers and the producers.

By-Products of Certain Food-Processing Industries

As indicated above the development of food industries helps to conserve food-stuffs and maintain a more or less even price throughout the year. Apart from these, the food industry provides by-products of considerable value which are lost when unprocessed foods are consumed in individual homes. For instance, in the canning of orange juice in America a number of by-products are obtained. The oranges are processed by washing, halving, pressing out the juice mechanically which is deaerated under vacuum, flash-pasteurised at 196°F. and then bottled or canned with or without concentration under reduced pressure. From this industry the peelings of the oranges are obtained which are pressed to give the orange oil which is an important flavouring agent. The pulp is preserved in barrels with preservatives for preparing squashes. The residue of the orange peelings is dehydrated into a valuable stock-feed by drying it in a rotary dryer. There is a proposal to mix it with wheat flour also for human consumption. Attempts are also being made to utilise the aqueous portion separated from the orange oil. This is being concentrated by a triple effect evaporator to a molasses-like substance which is used as a mixture in stock-feed. There is also a proposal to ferment it to alcohol or to grow yeast for either human consumption or for cattle feed. Pectin is also being produced from the peelings. This is a well-known product of considerable value.

It will be observed from the above that in the orange-processing industry not only the orange juice is made available to the consumers in a readily consumable condition but it also provides a variety of important by-products which would have been lost if the oranges were consumed in individual homes.

It may be stated in passing that orange juice has been sought to be concentrated by freezing out the water as ice and centrifuging (Krause-Linde process). The process does not seem, however, to have yet been used industrially in America. Beer has also been sought to be concentrated similarly.

Another processing industry in which a number of useful by-products can be obtained is that of fish. In such an industry the fish is usually filleted and then used for canning, refrigeration, etc. The scales are often converted into glue. The fish offal including rotten fish is dried in a rotary dryer to give a good protein meal for stock and poultry feed. Before drying, the offal is treated with live-steam in a horizontal rotary cylinder. The material is squeezed in an expeller which expels all the fat-water mixture. This is then separated. The fat is used and the water solution is evaporated in vacuum to give a paste rich in "vitamin B" which is a good poultry feed. The livers, if of right quality, are processed for the production of liver oil. Shark liver oil is subjected to molecular distillation in America to give a very potent vitamin A concentrate.

It will be seen from the above that a fish-processing industry not only provides fish to the consumers in a readily edible condition, which entails practically no drudgery in the home, but it also provides a number of valuable by-products which are lost when whole fish is bought and prepared in individual house-holds.

Similar remarks apply to meat and vegetable processing industries.

Food and Nutrition Policy

Researches on food and nutrition must consistently be applied to the practical problem of improving the nutritional level of the people. All scientific work must have as its goal the increase of the sum of human happiness. Our country is pre-eminently one where hardly a fraction of accumulated knowledge has been applied in various fields of national welfare. Since nutrition is the first and basic element for life and health it should have the highest priority for consideration. It may be interesting in this connection to recall the experience of a British medical officer who found, curiously, that on removing certain working class families from slums to better hygienic quarters the health of their children deteriorated. This apparent anomaly was traced to the fact that these families had to spend a larger proportion of their income on the rent of their apartments than they had done before and consequently the money available for food was less. The deficiency in food was dramatically felt. As Gowland Hopkins once remarked, among all the environmental factors which determine health and vigour, nutrition was the most important. The aim of modern civilization should be to achieve the highest standard of health of which the individual is capable within the limits set by heredity.

The scientific principles of nutrition have been applied with a great measure of success during the recent war in some of the Western countries. In the U.K. rationing has actually improved the nutritional level of the poorer sections of the population. In fact, it has been stated that the average stature of the British boy or girl of to day is significantly higher than before the war. This is attributed to the fact that the whole organisation for nutrition in England has been run under effective scientific guidance. At the beginning of the War, the scientific organisation of the Ministry of Food carried out an estimate of the food production in calories, protein, etc. in normal times and compared it with the nutritional requirements of the people of Britain in respect of all the nutritive factors. The next question was to meet the deficit from increased home production and from imports. It was decided to stimulate production of energy-yielding food-stuffs like wheat and potatoes in Great Britain in order to save shipping space and to import largely; concentrated and protective

foods like dried egg powder, milk powder, orange juice etc. Milk was first given to children and expectant and nursing mothers, who formed the priority groups, and then, if available, to adults. Milk was given free to tens of thousands of children and at cheap rates to other children and expectant and nursing mothers. A national flour was introduced, as has been mentioned earlier, which was richer in B vitamins, iron and calcium than the pre-war flour. Communal feeding was introduced (as had been done long ago in the Soviet Union) through factory canteens and through the so-called British Restaurants which catered for the public. Cheap meals, nutritionally balanced under scientific guidance, were provided in these communal feeding centres. Special capsules containing vitamins A and D were provided for expectant and nursing mothers. Rationing for different age groups was based on nutritional considerations and provided optimum nutrition according to modern accepted nutritional standards. Constant checks were kept on the nutritional status of different groups of the population produced by the rationing system and other food and nutritional measures. Periodic reports of this status regarding representative groups of the British population were regularly received by the Ministry of Food which examined these reports and kept their administrative measures in constant review in the light of these. A group of workers was appointed at the Oxford University to constantly look after the nutritional status of the people from the clinical point of view as affected by current rationing and other food measures.

The way that the Ministry of Food has handled food and nutrition problems of the U.K. during difficult times by taking and implementing scientific advice all along is an example to many other countries. All these scientific nutritional measures, however, could not have achieved much had not the Government sought constantly to grow and import all the food materials that were needed. The production of food in the U.K. increased by over 50% in certain commodities. The policy which was principally responsible for both the success of the "Grow More Food Campaign" and the stabilisation of the prices of staple food-stuffs was the policy of direct subsidy to the food-growers including producers of wheat, potatoes, meat, milk etc. In one year alone over two hundred crores of rupees have been given in direct subsidy to the food growers. The result has been that more food has been produced and the prices of none of the staple food-stuffs have risen higher than 25% above the pre-war level. Potatoes were sold at London at $1\frac{1}{4}$d. per lb. This could not have been achieved by price control decrees alone. Similarly constant attention was paid to the importation of concentrated food and protein food. The whole food position of the U.K. was under constant review and the Government made every effort to see that all the food required for the entire population, both civilian and army, on nutritional considerations was actually available to the people. The results have shown that the Government by its efforts succeeded in this objective.

The nutrition policy which has achieved so much success in the U.K. can be followed with suitable modifications in this country. The first task is to estimate the requirements of food by the people of India in the light of optimum nutritional considerations. In an article published in March, 1944 (*Science and Culture,* Vol. IX, p. 375) I had given the following requirements for an adult which may be considered nearly optimum. The relative proportions of different ingredients of this diet may of course be varied within certain limits and also some variations in the nature of the cereals according to local supplies may be permitted:

Unmilled or lightly milled rice	10 oz.
Whole wheat	6 oz.
Pulses	1 oz.
Sugar	2 oz.
Milk and milk products	10 oz.
Fish and meat (or extra milk products for vegetarians)	1 oz.
Non-leafy vegetables	5 oz.
Green leafy vegtables	5 oz.
Fats and oils	2 oz.
Fruits	3 oz.
Eggs	one or two

Such a diet would supply roughly the following nutritive elements:

Carbohydrate	470 g.
Fat	75 g.
Protein	96 g.
Calcium	1.1 g.
Phosphorus	2 g.
Iron	31 mg.
Carotene	4 mg.
Vitamin A	1.6 mg.
Thiamin	2 mg.
Riboflavin	1 mg.
Vitamin C	90 mg.
Calories	2,780

This would entail an annual production in India of the following:

Cereal grains (mostly rice and wheat)	65 million tons
Pulses	16 ,, ,,
Sugar and "Gur"	8 ,, ,,
Milk	40 ,, ,,
Fish and meat	16 ,, ,,
Nonleafy vegetables	20 ,, ,,
Green leafy vegetables	20 ,, ,,
Edible oils	7½ ,, ,,
Fruits	12½ ,, ,,
Eggs	15 billion

Sufficiently accurate statistics are not available about the present production of many of these food-stuffs. But it is clear that increased production will be necessary in each of these items if these nutritional requirements have to be met. It should be stated that the diet indicated above does not in several respects come up to the standards laid down by the National Research Council of America, which however may be a little too high.

Regarding vitamins, owing to the great developments in their manufacture, it is now possible to provide synthetic or concentrated vitamins to large masses of the population at a reasonable price. The targets of the annual production of vitamins in India may be tentatively indicated as below:

Carotene	300 tons
Vitamin A (concentrate)	150 ,,
Riboflavin	300 ,,
Thiamin	300 ,,
Nicotinic acid	3,000 ,,
Vitamin C	7,500 ,,
Calciferol	1.5 ,,

Indian diets are also usually low in calcium and the following targets may be given for the annual production of assimilable calcium and phosphorus compounds calculated as calcium and phosphorus 1.2 lac tons and 2 lac tons respectively.

The above would indicate the lines on which development should take place leaving room for readjustment of figures of production.

The provision of vitamins and minerals in tablets is no longer a subject for humorous comment but is already being practised in America and appears to be a desirable development from the standpoint of the improvement of the general level of nutrition. Calories and proteins are required in fairly large amounts but vitamins and minerals are required in relatively small amounts. The last two are also not often present in adequate quantities in the food we consume. It is possible to provide the vitamins and minerals in the form of tablets or capsules. If the major portion of the requirements of these food elements can be met from the manufactured materials, leaving the rest to be provided by the natural diet, it seems entirely logical to adopt this system instead of leaving it to chance that the ordinary food would provide adequate quantities of these nutritive elements. This may entail a slight change in our food habits, but food habits have changed during centuries. It should, however, be stated that in spite of the synthetic vitamins natural food-stuffs are still needed to provide probably still unknown vitamins. But there is no reason why we should not take the more important synthetic vitamins daily thereby ensuring an adequate supply of these particular substances. The chief aim should, however, always be to provide adequate natural food to all people.

The problem of food before India and also other oriental countries is colossal. How ill-fed Asia and Africa are compared with other continents would be indicated from the following table taken from Marrack, which gives the distribution of world's food supplies among the different continents:

Continents Food supplies	Europe	Asia	North America	Latin America	Africa	Oceania
Cereals and other food-stuffs	11.2	21.6	21.1	5.8	2.5	1.6
Meat	45.7	5.6	29.7	11.9	3.1	3.7
Per cent of world's population	25.9	52.5	6.5	5.7	6.7	5.5

It will be noticed that the consumption of practically all food-stuffs in Asia and Africa is frightfully small compared with the consumption in Europe and America although Asia and Africa are far more populated than the other continents. This is indeed a shocking situation.

Science and technology have placed plenty within the reach of all the people of the world. The fact that hundreds of millions of people throughout the world are on a low subsistence level is a slur on our civilization which we can remove only by establishing a social and political order which will ensure that it is not the income of the individual but his physiological needs that will determine the food that he eats. Until this social change is brought about, humanity will continue to wander in the wilderness, and our long night wll never dawn.

THE FOOD PROBLEM OF INDIA*

It has been estimated that to feed a population of 400 million India needs an increase in cereals to the extent of 10 per cent, in pulses to the extent of 20 per cent., in fats and oils to the extent of 250 per cent, in fruit 50 per cent, in vegetables 100 per cent., in milk 300 per cent., and in fish, flesh and eggs 300 per cent. These figures are staggering, because first of all these deficiencies have to be made up for the proper nutrition of the existing population, and a further increase has to be assured to meet the demands of the increasing population. For instance, to provide adequate nourishment for a population of 500 million in 1960, the production of cereals will have to be increased by 37.5 per cent., pulses by 50 per cent., fats and oils by 337.5 per cent., milk, fish, flesh and eggs by 400 per cent. With such deficiencies in food resources, it is not surprising that the Nutrition Advisory Committee have found from the results of actual "surveys of both typical urban and rural groups that the calorie intake of some 30 per cent. of families is below requirements and that even when the diet is adequate it is almost invariably unbalanced, containing a preponderance of cereals and insufficient protective foods of high nutritive value." There cannot be any disagreement on the point that "malnutrition promotes a state of ill-health and lower physical efficiency, short of actual disease ; which are perhaps more important because more widespread than disease itself." Therefore, the Nutrition Advisory Committee correctly lays stress on the fact that "freedom from disease is one thing, abundant health is another" and "the goal to be aimed at is the creation of a healthy and vigorous population."

Solution of the problem

The solution of the complex problem of providing adequate food for our population lies in the increase of the supply and, if possible, the decrease of demand.

On one extreme we have those who maintain that India is greatly over-populated and that her food resources have not kept pace with the rise of population and are progressively falling short of the minimum requirements and, therefore, "our present need is that the growth of population should be checked and even its decline welcomed!" They say: "Judged from any point of view a check on the growth of the population of India is an urgent necessity" (Chand). There can be no doubt about the urgency of such an attempt as it would bring about a measure of relief and allow scope for adjustment. A stationary population for some years would avoid "futility and frustration" which the present situation strongly suggests.

* Extracted from the General Presidential Address by Prof. M. Afzal Husain to the Thirty-third Session of the Indian Science Congress, Bangalore, 1946.

However desirable, a check on the growth of population may be, it is difficult to attain. Nevertheless, we may look at this problem from another point of view.

The United Nations have now accepted the responsibility for meeting the food requirements of all people. They must, therefore, determine the production of food and control its distribution. We are already hearing of world's wheat pools. The necessary corollary to this responsibility is that the United Nations will have to watch the population trend of various countries. What will be the attitude of the nations with a low or controlled birthrate towards another nation with an uncontrolled and very high birthrate? Will not the United Nations Organisation be justified in exercising some control over population? Having accepted membership of the community of nations, India will have to fall into line with the rest of the world. The solution of the population problem is not easy and at any rate it will be many years before a satisfactory solution can be found. In the meantime an increase in population will continue.

On the other hand there are those who firmly believe that "Nations can live at home" (Wilcox), and see in the development of the modern science of agrobiology the possibility of a manifold increase in the produce from land. They claim that the problem is not of *over-population* but of *under-development* of the natural resources and inadequate utilisation of human knowledge to develop these resources. For instance, Wilcox places the theoretical limit of the yield of wheat at 171 bushels and of potatoes at 1,330 bushels, while the average in U.S.A. is only 8.4 per cent. of this 'penultimate' limit in the case of wheat and 8.6 in the case of potatoes.

Neither the policy of population reduction nor the magic wand of agrobiology can bring forth immediate results. The time factor is important. The Bengal Famine and insecurity of the food position are clear warnings. A sound policy would be to base our programme on the results previously achieved and attempt to evolve a scheme of increased food production from existing resources, leaving future enhancement of production for the increased population.

Unfortunately, determining food requirements by calories has produced an attitude more in favour of quantity than quality, and this has made it difficult to arrive at a scientifically correct food policy. Cereals have assumed unnecessary importance at the expenses of 'protective' foods. All those who have studied the food problem of India have emphasized this point. Colonel Macay held that with a low protein consumption deficiency in stamina, moral and physical, must be expected. According to John Russell, the well-balanced diet for India "does not require more but less cereal than at present, but it includes more of everything else, especially vegetables, fruits and milk, and one great need for the food supply is to increase the production of these three." He advocated an increase in the yield of staple crops so as to liberate land for the cultivation of supplementary foods. India's ill-balanced diet, which has led to extensive malnutrition, is a far more serious national problem than any mere deficiency in the quantity of food. The population is degenerating in physique and in stamina. How else can one explain the curious phenomenon that lakhs died in Bengal without attempting to obtain food by fighting for it! To arrive at a correct appreciation of the food situation, it is necessary to deal with the various constituents of the diets, and not talk of calories, however convenient the slogan may be.

Let us shake off the cereal mentality and the talk of carbohydrates, fats, proteins, minerals, vitamins and so on, and make an attempt to evolve a scheme of a

'balanced diet' containing as far as possible all the ingredients in their correct proportions.

Requirements of Carbohydrates

The present position is that over 72 per cent. of the carbohydrates of human food are derived from cereals, about 20 per cent. from sugarcane, and the balance mainly from pulses. India, with 90 per cent. of her cultivated area under food-crops and 64 per cent. under cereals, is short of rice and is barely self-sufficient in other cereals. In spite of an intensive "Grow More Food" campaign, increased production has not kept pace with increased demand, and India is seeking imports at least at the pre-war level. It does not seem likely that India will obtain rapidly enough such a phenomenal rise in her soil fertility, such colonization of vast tracts of land, such rapid extension of irrigation, as to make up the existing deficiencies and provide for the future population, from a cropping scheme built round 64 per cent. area under cereals. In the circumstances India must produce, per acre, quantities of carbohydrates much in excess of what cereals can possibly yield. Because, if the required quantities of fuel foods can be produced from a smaller area, it would be possible to release land for the increased production of pulses, fats and oils, and "protective" foods of vegetable and animal origin, in which India is greatly in deficit. Tubers will satisfy this requirement.

In all countries where the population has increased, cereals have been increasingly replaced by tubers. For instance, in Germany, area under potatoes is 25 per cent. of that under all cereals. In England, it is 17.8 per cent. Even U.S.S.R. has 17.6 million acres under potatoes. In Java, one of the most thickly populated parts of the globe there has been, since 1916, a great increase in the cultivation of cassava and sweet-potato. In many countries of Europe potato shares with cereals, more or less, on a basis of equality, in the carbohydrate supply of the human diet. Even in the United States, in spite of the availability of land, the ration of cereals and potatoes in the diet of a household of the lowest income is 79.8 to 64.4.

Food Value of Tubers

As regards their food value: reduced to the same standard of moisture, tubers are richer in carbohydrates, mineral matter and calcium than cereals ; they are, however, poorer in proteins and deficient in fats. The great advantage of tubers over cereals is the yield per acre. If the average yield of rice and wheat in India be taken as 10 maunds per acre (although it is less), and the average yield of potatoes be taken as 75 maunds per acre (although it is more than 100 maunds), the per acre yield of various constituents of food will be very much higher in the case of tubers, except fat in potato and protein in cassava.

With a reasonable standard of cultivation, a yield of 200 maunds per acre is not difficult to attain in the case of potato, sweet-potato and cassava. With this yield the potato will provide a quantity of carbohydrates at least four times that of wheat, and sweet-potatoes and cassava about five times.

The superiority of rice and wheat in contrast to tubers is their high protein content. There seems no reason why India should persist in obtaining her protein supply from cereals. She must obtain the various ingredients of diet from sources which can be produced most efficiently and economically. In other words carbohydrates must be obtained mainly from tubers and cereals if possible

in equal proportions ; proteins from pulses and animal sources such as milk, fish, flesh and eggs ; fats and oils from milk and oil seeds ; minerals, vitamins and other ingredients from such sources as supply them most economically.

In addition to providing large supplies of carbohydrates, minerals, calcium and phosphorus per acre, tubers can be used as fodder for livestock, as a source of starch for food products, such as biscuits, and a raw product for the manufacture of dextrine, glucose and sizing for the textile industry. In these respects they outstrip cereals. From the agricultural point of view, they loosen the lower strata of soil and lead to soil improvement. Potatoes respond to better cultivation and provide increased occupation for the farmer. There are some varieties of tubers that yield two and three crops a year, in which case the yield per acre is exceedingly high.

The greatest obstacle in the extension of the area under potatoes in India is the non-availability of sound healthy seed in adequate quantities, at the right time and at a reasonable price. The crop grown in the plains gets diseased and, therefore, seed has to be brought from the hills or imported from abroad. Researches have shown that healthy seed can be produced in India, and according to Burns, "given disease-free seed-potatoes and suitable manuring, the production of potatoes on the existing acreage can be doubled." Steps have been taken by the Imperial Council of Agricultural Research for the production and distribution of healthy seed. There are vast areas which provide suitable soil and climatic conditions for potato cultivation and in many parts of India two crops can be raised in a year.

SWEET-POTATO

If potato is the tuber of the cooler regions, sweet-potato may with greater justification claim to be the tuber of the warmer regions of the globe. If potato is the tuber of West, sweet-potato is the tuber of the East. "The Chinese cultivate sweet-potato on a very large scale, and it enters into their diet, in some parts even more than rice." During 1943 the U.S.A. had 900,000 acres under sweet-potatoes, mainly in the Southern States. Some varieties of sweet-potatoes are only three-month crops. Even two crops a year, each yielding 200 maunds of tubers, grown over a moderate area, would convert Bihar and Bengal from deficit to surplus provinces, not only for carbohydrates but by releasing area for fodder, which will also increase the supply of milk.

Sweet-potato has this advantage over potato that it can be grown from stem-cuttings and the seed problem, the greatest obstacle in the extension of area under potato, does not arise. Again its demands for soil, manure and irrigation are not exacting either.

PROPOSALS

If India could grow cereals and tubers in the same proportion as the pre-war Germany, i.e., in the proportion of 4: 1, India could supply in full her present requirements of carbohydrates from an acreage equal to 60 per cent. of what is under cereals now. Even if 10 per cent. of the acreage now under cereals be diverted to tubers, India's carbohydrate supply will be increased by 33 per cent. By following such a policy, land could be released for pulses, oil-seeds, fodders and a more balanced diet obtained.

The proposal I place before you is that, if the area under cereals is reduced from the present 64 per cent. of the total sown to 45 per cent. or so, and of the area

thus released, 5 per cent. of the total sown be planted with tubers, and the acreage of pulses be increased by 20 per cent, the out-turn of carbohydrate will be much in excess of the present quantity. I have taken tubers as an instance of high-yielding crops. Equally satisfactory results can be obtained from plantains, which yield over 200 maunds of fruit per acre, and produce as much carbohydrate as sweet-potato or cassava with 100 maunds to the acre. They are also decidedly richer in proteins. Another high-yielding crop is carrot, which has the added advantage of being a rich source of carotene.

Requirements of Fats and Oils

India's requirements of fats and oils have been placed at 250 per cent. in excess of the available supply. The area released from cereals could permit the acreage under edible oil-seeds being doubled. This would also double the quantity of concentrates for feeding the milch-cattle, and if a reduction in the number of bullocks can be brought about simultaneously, as suggested later on, there will be a further improvement in the food resources of milch-cattle. The introduction of Soya bean, a legume rich in oils, will greatly enhance the supply of edible oil. In planning the nutrition of the whole world, the advisability of exporting oil-seeds from a country grossly deficient in fats and oils, will, we hope, be determined by the Food Advisory Organization of the United Nations.

Protein Deficiency

Deficiency in total proteins, and more particularly in the proteins of high biological value, is India's most serious nutritional problem. This deficiency may not manifest itself in mortality and disease, but is evident in the slow rate of growth, reduced size of body, lack of efficiency and vitality. That this is actually the case is abundantly manifested by the condition of both men and cattle. Dr. Burns has correlated the amount of food and body-weight in cattle of the different regions of India, and Prof. Radhakamal Mukherjee has made similar studies in human groups. It is apparent that where cattle are ill-fed and small in size, and milk production per head of human population is low, the human physique is poor. Average live-weight of cattle and man is fairly closely correlated.

Pulses and cereals are the chief source of vegetable proteins. Reduction in the area of cereals will reduce the quantity of proteins of this source slightly, but a 20 per cent. increase in pulses will make up the deficiency. It is, however, the increase of proteins of high biological value, which is India's greatest need.

The Nutrition and Food Management Committee of the FAO have recognised that "the primary objective of the nations united in the Food and Agriculture Organization is to raise the level of nutrition throughout the world, to ensure not only that all people are freed from the danger of starvation and famine, but that they obtain the kind of diet essential for health." Our Food Policy should aim at 'abundant health', and our goal should be the creation of healthy and vigorous population, able to shoulder the burdens of peace and war.

Protective Foods

Let us now deal with the foods of animal origin "protective foods" and proteins of high biological value, provided by fish and flesh, eggs and milk. The requirements of these foods for 400 million human beings is estimated at 300 per cent. over and above the present supply.

The most important of the food resources of this category are fish. The extensive waters around the coast of India, vast estuarian areas, numerous rivers and canals, lakes and tanks provide almost unlimited possibilities for the production of fish. Fish may be described as the food ready-made for man to collect. The neglect to develop, nay even to control, the fisheries in India has been colossal. It is only under the stress of war-time food scarcity that the necessity of developing this valuable source of food has been recognised. It is encouraging to find that several Provinces and States, as well as the Central Government have taken steps to develop the fishery resources of the country. Programmes of development include all aspects of the fish industry, and teaching and research. We can look forward with confidence to the full development of this source of food. An abundant and cheap supply of fish will solve the problem of a balanced diet for the enormous rice-eating population. No effort should be spared to develop fisheries.

Sheep, goats, pigs and poultry are well-known sources of food. The Imperial Council of Agricultural Research are financing research on these animals, with a view to improve breeds and increase the quantity of food produced from these sources. Among the smaller animals, a useful source of wholesome flesh is the rabbit. It multiplies very rapidly and grows quickly. In other countries rabbit-breeding is an important industry, and it is a pity that in India nothing has been attempted so far and this excellent source of very good food is being ignored.

Investigations carried out in America indicate the importance of wild life. It has been shown that where marshes have been reclaimed for cultivation, the benefit gained has not compensated for the loss sustained, through the destruction of water-fowl. We have approximately 200,000 square miles of forests. Can they not be stocked with eatable birds? There is immediate need for a thorough survey and population study of the wild life of India as a preliminary to a national planning of game improvement.

Conclusion

A national crop planning should be based on the best and most efficient utilisation of land and other resources for social needs. The first social need is food. It is possible to evolve, for the various parts of the country, cropping schemes which will result in greater production of carbohydrates from smaller areas, than is the case at present. In any such scheme tubers will play an important part, and the area under cereals will have to be reduced. Acreage released from cereals can be devoted to pulses, oil-seeds, fodders, an increased production of which is necessary for obtaining well-balanced diet. The increased food for milch-cattle, both in roughages and concentrates, which will result from such a cropping scheme, will make up our existing deficiencies in milk—a most necessary 'protective food.' The introduction of more legumes, i.e., pulses as well as fodders, will enrich our soils. A reduction in the number of bullocks by encouraging the use of tractors and motor transport, and the introduction of machinery driven by cheap electric power, will release much fodder and enable us to improve our breeds of milch-cattle, with a consequent increase in milk production.

All this is possible and we have the knowledge to do it, but as the Hot Springs Conference stated—"It requires imagination and firm will on the part of each government and people to make use of that knowledge."

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INVESTIGATIONS ON MUCUNA SEEDS
PART II: BIOLOGICAL VALUE OF PROTEINS OF THE SEEDS OF *MUCUNA UTILIS*, WALL.

S. N. SARKAR AND A. C. BOSE
From the Biochemical Laboratory, Dacca University, Dacca.

(Received for publication, April 10, 1945)

In a previous communication (1) the biological values of proteins of the seeds of *Mucuna pruriens*, DC by the balance sheet method as well as the growth method have been reported. The present work deals with the determination of biological values of the seed proteins of *Mucuna utilis*, Wall by both methods. These seeds are shining black in colour and over twice as large as those of *Mucuna pruriens*, being sometimes offered for sale as substitutes.

A large quantity of these seeds were decorticated and ground to a fine powder in a flour mill. This powder served as the material for study in this investigation.

EXPERIMENTAL

The seeds were found to contain moisture 12%, protein 27.81%, ether extract 4.49% and ash 3.3%.

The biological value of the seed proteins was first determined by the balance sheet method and the technique employed was the same as that followed by Basu *et al* (2). The metabolic nitrogen, however, has been calculated as being directly proportional to the food-intake.

The composition of different diets used is given in Table I.

TABLE I

Composition of Different Diets

Constituents	N — free diet	per cent protein content of diet. 6.25%	10.5%	15%
Decorticated mucuna powder ...	...	225.0	378.0	510.0
Chopped sugar	90.0	90.0	90.0	90.0
Butter fat	100.0	90.0	83.2	76.0
Cod liver oil	20.0	20.0	20.0	20.0
Salt mixture	50.0	50.0	50.0	50.0
Ca-Carbonate	8.0	8.0	8.0	8.0
Starch (B.D.H.)	735.0	520.0	371.0	219.0

TABLE II

Experiments with Nitrogen-free ration

(figures for intake and excretion represent daily averages)

Rat No.	Body weights in g.	Food intake in g.	Urinary nitrogen in mg.	Metabolic nitrogen in mg.	Metabolic nitrogen per g. of food in mg.
1M	155.0	8.0	29.10	11.90	1.86
2F	235.0	8.5	37.60	14.96	1.76
3F	210.0	7.0	33.40	8.02	1.15
4M	153.0	9.0	20.13	19.41	2.16
5M	160.0	12.3	24.44	21.45	1.74
6M	112.0	9.8	20.66	20.50	2.09
7F	206.0	10.0	21.78	22.03	2.20
8M	185.0	9.0	25.81	21.50	2.72

Data obtained in experiments with the nitrogen-free diet are given in Table II. In Tables III and IV are given relevant data for metabolism experiments with diets containing 6.25% and 10.5% of seed proteins. Experiments were carried out at 15% level also but here as in the case of seeds of *Mucuna pruriens*, the condition of rats was not normal and as such data for this experiment are not reported.

A perusal of data submitted in various tables show that the biological value by the balance sheet method at 6.25% level of protein intake is 56 and that at 10.5% is 43 only. The percentage digestibility figures, however, are fairly high, 81.9 at 6.25% level and 82.4 at 10.5% level.

In view of interesting results obtained above it was thought desirable to study whether the proteins present in seeds of *Mucuna utilis*, have any growth-promoting property. For this, experiments were carried out with young growing albino rats. The technique followed was that adopted by Basu *et al* (loc. cit).

In the case of the seed proteins of *Mucuna pruriens* it was shown that, at 15% level of protein intake, there was considerable disturbance in the system of experimental rats and as a result there was practically little growth in rats at this level. Moreover after some time they began to show a loss in the body-weight. The proteins of *Mucuna utilis*, Wall, have high biological value (by balance sheet method) at 5-6% level but at 10-11% level the value was much lower which points to some abnormality in the system of animals and suggest that these seeds are much more toxic than those of *Mucuna Pruriens*. This view has been further substantiated by the present experiment.

TABLE III

The biological value of Mucuna utilis seed proteins at 6.25% protein level

(figures for intake and excretion represent daily averages)

Rat No.	Body weight in g.	Food intake in g.	Nitrogen intake in mg.		Urinary Nitrogen in mg.			Fæcal Nitrogen in mg.			Biological value	Mean B.V.
			Total	True	Total	Endogenous	True	Total	Endogenous	Exogenous		
1M	156.0	11.10	111.0	88.65	67.2	29.1	38.10	13.0	20.65	22.35	57.0	
2F	237.0	13.61	136.1	116.06	89.0	37.6	51.10	13.99	23.95	20.01	55.7	
1M	153.0	11.86	118.6	99.22	62.13	20.13	12.30	15.01	25.63	19.39	57.1	56
5M	159.0	11.53	115.3	88.81	61.95	21.14	10.51	16.51	20.05	26.19	54.1	
6M	111.0	9.67	96.7	80.93	56.1	20.66	35.11	35.98	20.21	15.77	56.2	

TABLE IV

The biological value of Mucuna utilis seed proteins at 10.5% protein level

(figures for intake and excretion represent daily averages)

Rat No	Body weight in g.	Food intake in g.	Nitrogen intake in mg.		Urinary Nitrogen in mg.			Fæcal Nitrogen in mg.			Biological value	Mean B.V.
			Total	True	Total	Endogenous	True	Total	Endogenous	Exogenous		
1M	151.0	5.50	92.10	78.07	61.07	20.13	13.91	26.21	11.88	11.33	43.7	
5M	162.0	6.10	102.50	81.82	70.52	21.11	16.08	28.29	10.61	17.68	45.7	
6M	111.0	5.00	81.00	73.12	61.60	20.66	13.91	21.03	10.15	10.58	40.2	43
7F	206.0	5.10	85.69	70.16	63.27	21.78	38.19	26.75	11.22	15.53	45.1	
8M	186.0	7.10	119.30	89.81	78.0	25.81	52.19	18.77	19.31	29.16	41.9	

It has been observed that all the rats at 15% level and 10.5% level are unable to grow and die gradually. The observations made in this connection are recorded in Table V.

TABLE V

The performance of rats at 15% and 10.5% protein level

Rat No.	Level of protein intake	Time of Death	Amount of protein consumed. in g.	Loss in body weight in g.
9M	15%	8th day	6.7	—7.0
8F	15	9th day	7.2	—7.5
7M	15	10th day	7.0	—9.0
6M	10.5	9th day	5.1	—8.5
5F	10.5	10th day	5.5	—10.5
4M	10.5	12th day	5.7	—9.5

Coming to the 6.25% level it is seen that rats were able to maintain their body weights somehow after an initial decrease to some extent. The table below gives an idea.

TABLE VI

The performance of rats at 6.25% protein level

Days of feeding	Rat No. 3M		Rat No. 2F		Rat No. 1M	
	Body weight in. g.	Protein intake in g.	Body weight in g.	Protein intake in g.	Body weight in g.	Protein intake in g.
0	36.5	2.2	33.5	2.0	13.5	2.5
7	33.5	1.6	31.5	3.8	10.5	4.8
11	29.5	6.9	27.0	6.0	31.0	7.1
21	28.0	9.0	26.0	8.1	32.5	9.6
28	28.0	11.1	25.5	10.2	33.0	12.0

It is clear from the above table that the body-weights of rats at 6.25% level of intake after coming down to a lower value remain more or less stationary. In view of this stale-mate condition the experiment was not continued further.

SUMMARY

The biological values of proteins of the seeds of *Mucuna utilis*, Wall, by the balance sheet method at 6.25% and 10.5% levels of protein intake are 56 and 43 respectively.

The mean digestibilities at 6.25% and 10.5% levels are 81.9 and 82.4 respectively.

The seed proteins have practically no growth promoting value.

These seeds appear to be more toxic than those of *Mucuna pruriens*, DC.

Further work is in progress.

Our best thanks are due to Prof. J. K. Chowdhury for his kind interest.

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BIOLOGICAL VALUE OF PROTEINS OF PALMYRA FRUITS (*BORASSUS FLABELLIFER*, LINN)

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(Received for publication, April 10, 1945)

The juice of the ripe palmyra fruit (Bengali-*Tal*) and the edible portions (*Talsans*) of the green fruit are common articles of food in Bengal. It was thought interesting to examine the chemical composition of the juice of the ripe fruit and the 'talsans,' and determine the biological value of the proteins present.

EXPERIMENTAL

The outer coarse covering of the ripe fruit was peeled off and the pulp was pressed to yield a thick syrupy yellow juice. The juice was evaporated to dryness on a water-bath. The mass was then powdered as far as possible and preserved in a refrigerator.

The outer semi-hard white covering of 'talsans' was removed with a knife. The soft mass including the fluid inside was then dried on a water-bath. The dried mass was as before powdered and preserved in the refrigerator.

The composition of the samples are given in Table I.

TABLE I

Constituents	Dried juice of ripe fruits	Dried green fruits
Moisture	15.80%	21.00%
Total protein	4.20	7.00
Total nitrogen	0.67	1.12
Ether extract	0.50	0.30
Carbohydrate, etc.,	71.92	65.19
Ash	4.58	3.51
Calcium	0.07	0.05
Phosphorus	0.15	0.297
Magnesium	0.098	0.10
Silica	0.02	0.06

Methods recommended by the Association of Official Agricultural Chemists (A.O.A.C.) were used in general.

The biological values of proteins of the edible portions of green as well as ripe fruits were estimated by the balance sheet method. The technique followed for these determinations is the same as that followed by Basu *et al* (1). The metabolic nitrogen, however, is calculated as being directly proportional to the food intake.

The composition of diets is given in Table II. In Table III are given the results of experiment on nitrogen-free diet. The results of metabolism experiments with proteins of fruits are given in Tables IV and V. In Table VI, the percentage digestibility values are recorded.

TABLE II

Composition of diets.

Constituents	N-free diet.	Protein content —3.19% Ripe-palm diet.	Protein content —3.0% Green-palm diet.
Palm powder	...	762.0 g.	429.0 g.
Chopped sugar	90.0 g.	90.0	90.0
Salt mixture	50.0	50.0	50.0
Butter fat	100.0	73.0	98.0
Codliver oil	20.0	20.0	20.0
Ca- carbonate	8.0	8.0	8.0
Starch (B.D.H.)	735.0	0.0	308.0

TABLE III

Experiments with Nitrogen-free ration.
(figures for intake and excretion represent daily averages)

Rat No.	Body weight in g.	Food intake in g.	Urinary nitrogen in mg.	Metabolic nitrogen in mg.	Metabolic nitrogen per g. of food in mg.
1M	155.0	8.0	29.10	11.90	1.86
2F	235.0	8.5	37.60	11.96	1.76
3F	210.0	7.0	33.40	8.02	1.15
4M	153.0	9.0	20.13	19.11	2.16
5M	160.0	12.3	21.41	21.45	1.71
6M	142.0	9.8	20.66	20.50	2.09
7F	206.0	10.0	21.78	22.03	2.20
8M	185.0	9.0	25.81	21.50	2.72

TABLE IV

The biological values of the proteins present in the juice of ripe palmyra fruit.

at 3.19% level of protein in-take.

(figures for intake and excretion represent daily averages)

Rat No.	Body weight in g.	Food intake in g.	Nitrogen intake mg.		Urinary Nitrogen mg.			Fæcal Nitrogen mg.			Biological value	Mean B.V.
			Total	True	Total	endogenous	True	Total	endogenous	Exogenous		
1M	158.0	1.59	23.43	16.88	36.8	29.1	7.70	15.09	8.54	6.55	51.4	
2F	235.0	6.30	32.15	25.17	50.2	37.60	12.60	18.07	11.09	6.98	50.0	
3F	210.0	5.90	30.11	22.28	41.0	33.1	10.60	14.62	6.79	7.83	52.4	52.2
4M	150.0	6.99	35.68	29.40	33.75	20.13	13.62	21.38	15.10	6.28	53.7	
6M	111.0	6.69	34.11	26.39	33.75	20.66	13.09	21.73	13.98	7.75	50.4	

TABLE V

The biological values of proteins present in the edible parts of green palmyra fruits.

at 3% level of protein intake.

(figures for intake and excretion represent daily averages)

Rat No.	Body weight in g.	Food intake in g.	Nitrogen intake mg.		Urinary Nitrogen mg.			Fæcal Nitrogen mg.			Biological value	Mean B.V.
			Total	True	Total	endogenous.	True	Total	endogenous	Exogenous		
8M	182.0	7.0	33.6	22.71	31.60	25.81	8.79	29.9	19.01	10.86	61.4	
7F	201.0	5.0	21.0	18.95	31.37	21.78	6.59	16.05	11.00	5.05	65.2	
5M	155.0	11.38	51.6	50.59	41.11	21.44	19.67	23.81	19.80	1.01	61.1	62.6

TABLE VI

The digestibility of ripe and green palmyra fruit proteins.

Protein level %	Rat No.	Food Nitrogen Intake	Food Nitrogen digested	Percentage digestibility	Mean
3.19	1M	23.13	16.88	72.0	
3.19	2F	32.15	25.23	78.5	
3.19	3F	30.11	22.28	74.0	76.8
3.19	4M	35.68	29.40	82.4	
3.19	6M	31.14	26.39	77.3	
3.0	The average percentage digestibility of 'talsans' from the green fruit ...				80

A perusal of the foregoing tables shows that though the protein content of the edible parts of green or ripe palmyra fruit is small yet the biological value and the digestibility figures are fairly high. The biological value of the proteins of the dried juice of the ripe fruit at 3.19% level of protein intake is 52.2 and that of the green fruit at 3% level is 62.6. The digestibility of the proteins of the ripe fruit juice is 76.8 whereas that of the green fruit is 80.

CONCLUSIONS

1. The digestibility of edible parts of the green palmyra fruit is greater than those of the ripe fruit.

2. The biological value of the proteins of edible portions of the green fruit is also greater than that of the proteins of the ripe fruit.

Our best thanks are due to Prof. J. K. Chowdhury for his kind interest.

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ON PLANT PHOSPHATASES. PART V. LEAF PHOSPHATASES—DISTRIBUTION AND GENERAL PROPERTIES

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Although considerable amount of work has been carried out on the phosphatases from plant materials such as cereals, tubers, etc., there is very little information available on the occurrence, distribution and characteristics of leaf phosphatase. As the leaf is an important organ of plants which is the seat of photosynthesis and respiration and in view of the fact that leaf or foliar analysis has become well established in recent years as a useful aid in determining nutrient requirements of a wide variety of crops the knowledge of the exact rôle played by the enzyme phosphatase in leaves, its variation during growth of plants and its relationship to photosynthesis, respiration and carbohydrate metabolism are of considerable importance in the study of plant physiology. The only work so far reported on leaf phosphatases is that of Ignatieff and Wasteneys (1) who studied the changes in the phosphatase occurring in the various parts of the Canada Wonder bean, potato, radish and wheat plants and its concentration at different stages of the life history.

Later Giri (2) determined the activity of phosphatase from plant materials such as cereals, pulses, tubers and leaves and has shown that the leaves are rich sources of the enzyme compared to cereals and other plant materials. In view of the wide distribution of the phosphatase in leaves a systematic investigation on the rôle played by this enzyme is desirable. The present paper relates to the results obtained on (*a*) the distribution of phosphatase in leaves of various plants (*b*) its variation with age and metabolic activity and (*c*) the characteristics of the enzyme.

2

Experimental

Determination of Phosphatase Activity.—The phosphatase activity determinations were carried out by estimating the amount of inorganic phosphorus formed when a known amount of the active extract or dry powder was allowed to act in the reaction mixture, containing buffer and sodium β—glycerophosphate after a known interval of time of hydrolysis at $35° + 0.2$C in an electrically controlled thermostat. Unless otherwise stated the reaction mixture always contained 10 cc. of 1% sodium glycerophosphate (Merck) solution 10 cc. of M/5 acetic acid—acetate buffer (*p*H 5.2) and the enzyme material, the total volume being made up to 25 cc. Thus the concentration of glycerophosphate in the reaction mixture is 0.4%.

After one hour's hydrolysis, the inorganic phosphorus was determined colorimetrically in an aliquot of the reaction mixture by the method of Fiske and Subbarow (3) after precipitating the protein by 10% trichloracetic acid. From the figure thus obtained the inorganic phosphorus formed in the total volume of the reaction mixture was calculated.

In this paper the "phosphatase activity" is expressed as mg. of inorganic phosphorus formed per gram of dry leaf powder.

"Soluble or Free" Phosphatase.—The phosphatase contained in the aqueous extract of the powder is designated as 'free' phosphatase. This enzyme was obtained by extracting the powder with ten times the quantity of water for one hour at the room temperature and filtering in a Buchner funnel.

"Total" Phosphatase.—The phosphatase contained in the whole seed powder is designated as "total" phosphatase. The activity of the total phosphatase was determined by weighing a known amount (0.1-0.2 g.) of the powder and allowing it to act on the substrate under exactly similar experimental conditions used for determining the activity of the "free" phosphatase.

Preparation of leaf material.—As the composition of leaves varies with age, type and the time of the day, it is desirable that the selection and sampling of the leaves are restricted to the leaves of the same age and type. The leaves were collected in the morning between 6 and 10 A.M., and the soil and dust adhering to the leaves were removed by brushing with stiff brush. The petioles were removed and discarded. The leaves were then cut into small pieces and quickly placed in a desiccator to dry. After drying, the leaves were then ground in a mortar. The resulting leaf powder was sieved twice through a 40 mesh sieve. This treatment was found to give a fair separation of the mechanical tissues from the softer tissues of the leaf, which are more easily pulverised. The mechanical tissues (veins and midrib) are considerably lower in phosphatase content than the rest of the leaf (as shown in Table VII) and were rejected on the ground that the composition of the active metabolic regions of the leaf should give the best indication of nutritional status.

Drying of leafy materials.—The selection of suitable method of drying of the leaves is very important, as the activity of the enzyme is considerably altered by the method of drying. The following methods of preparation of dry powder from the leaves were tried with a view to finding a suitable standard method for the preparation of leaf powder without any appreciable loss of the enzyme activity. The cut leaves were subjected to—

1. Drying at 50° in hot-air chamber ;

2. Drying in desiccator under vacuum over anhydrous calcium chloride. The leafy material was spread in thin layers on a filter paper.

3. Treatment with acetone. The fresh leaf material (10 g.) was treated with 20 cc. acetone, well macerated and filtered after five minutes through a Buchner funnel. The operation was repeated with 20 cc. acetone followed by treatment with 15 cc. ether.

The following table shows that (2) and (3) are the best methods. Method (1) brings about slight inactivation of the enzyme.

TABLE I.

Total phosphatase activity in leaves subjected to different methods of treatment for the preparation of dry leaf powder.

(10 g. of the leaves cut into small pieces were used).

Leaves	Drying at 50° for 1½ hrs.	Desiccation for 6 hrs.	Acetone treatment.
1. *Carica Papaya*	9.85	11.95	12.78
2. French Bean	51.8	59.8	60.00

Although the acetone treatment yields a slightly more active material, the higher activity may be due to the increase in concentration of the enzyme in the final product as a result of the removal of acetone soluble constituents from the leaf material. For purposes of quantitative study of the phosphatase activity of leaves during growth of plants, it is, therefore, desirable to select the desiccation method which does not affect the activity of the enzyme and at the same time the material obtained by this method contains all the constituents originally present in the leaf.

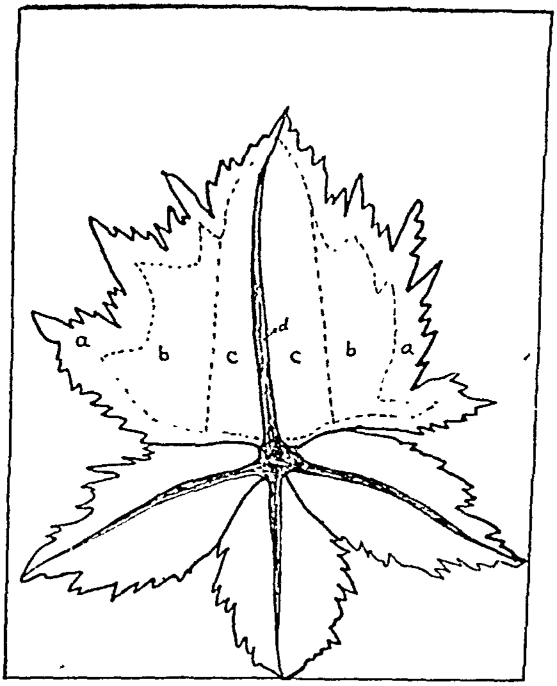

FIG. 1.
Carica papaya leaf.

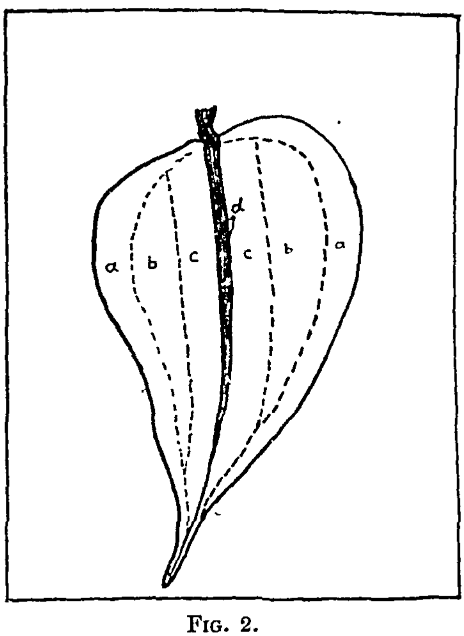

FIG. 2.
French bean leaf.

Influence of time of drying the leaves in vacuum on the activity of the phosphatase.

The time of desiccation of the leaves is of considerable importance, as the phosphatase activity is destroyed if the leafy material is not dried within a short time. Once the material has been dried, it does not lose its activity on storage.

In the following table are presented the variation in activity of the enzyme when the leaf is subjected to varying periods of desiccation, in vacuum.

TABLE II.

Influence of time of drying of leaf material (carica papaya) in vacuum desiccator on the activity of total phosphatase.

Time of desiccation.	Total phosphatase activity.
1. Desiccation for 12 hours (complete drying)	17.45
2. The same sample (1) after 12 hours desiccation kept in vacuum desiccator for 96 hours	17.45
3. Desiccation for 36 hours	12.78
4. Desiccation for 96 hours	2.95

It is clear from the above table that the longer the period of desiccation taken for complete drying, the more the inactivation of the enzyme in leaf powder. It is of interest to note that the phosphatase is inactivated to a greater extent under anaerobic conditions as can be seen from the following table which contains the results of experiments carried out in Thunberg tubes.

Two Thunberg tubes were filled with 10 g. of cut *Carica papaya* leaves. One of the tubes was immediately evacuated while the other was not. The two tubes were kept at room temperature (20-24°) for 1½ days and then the leaves were dried quickly in the same desiccator and the activities of the total phosphatase determined. The results are presented in Table III.

TABLE III.

Total phosphatase activity of leaves stored in presence and absence of air.

Expt. No.	Treatment of leaves.	Total phosphatase activity.
1.	(a) In presence of air	12.88
	(b) In absence of air	2.75
2.	(a) In presence of air with 1 cc. of toluene added	12.56
	(b) In absence of air with toluene added	2.63

It is clear from the above table that the storage under anaerobic conditions is very detrimental to the activity of the phosphatase. It is consequently necessary to minimise the time of drying in vacuum. The mechanism of destruction of the enzyme in leaves under anaerobic conditions of storage is worth investigating as it may throw light on the rôle of this enzyme in aerobic and anaerobic biological reactions.

Phosphatase activity of leaves of higher plants.—The leaves were dried in desiccator as described before and the phosphatase activity of the whole leaf powder was determined. In the selection of leaves, the age, maturity etc., were not taken into consideration. The object is merely to show the wide distribution of the enzyme in leaves. It is however necessary to find out if there is any relationship existing between the phosphatase activity of leaves and the natural order of the plants so that a rough classification of the plants based on phosphatase activity may be possible. Work in this direction is in progress.

TABLE IV.

Phosphatase activity of leaves of higher plants.

	Common name of the plant.	Botanical name.	Total Phosphatase.
1.	Paddy	*Oryza sativa*	7.5
2.	Cholam	*Sorghum Vulgare*	9.13
3.	Cambu	*Pennisetum typhoideum*	13.88
4.	Ragi	*Eleusine Coracana*	11.88
5.	Ragi straw	,,	1.15
6.	Peas	*Pisum Sativum*	18.15
7.	Barley	*Hordeum Vulgare L.*	20.85
8.	French bean (15 days)	*Phaseolus vulgaris L.*	51.1
9.	French bean (3 months)	,,	37.5
10.	Sanke gourd (tender leaves)	*Trichosanthes anguina*	23.78
11.	Pumpkin	,,	18.38
12.	Ash gourd	*Benincasa Cerifera Savi*	9.18
13.	Squash (tender leaves)	*Cucurbita Pepo L.*	21.65
14.	Lucerne	*Medicago Sativa L.*	6.9
15.	Plantain	*Musa Paradisiaca L.*	2.85
16.	Tomato	*Solanum Lycopersicun*	10.0
17.	Sugar cane	*Saccharum Officinarum L.*	9.3
18.	Castor	*Ricinus Communis L.*	7.93
19.	Papaya	*Carica Papaya L.*	24.3
20.	Margosa	*Melia Azadirachta*	6.5
21.	Akanda	*Calotropis Gigantea*	16.95
22.	Croton leaves (green variety)	*Codiaeum Variegatum*	27.8
23.	Brinjal	*Solanum Melongena L.*	14.18
24.	Lemon	*Citrus Medica L.*	6.25

The results presented in Table IV would show that the phosphatase is widely distributed in the leaves of all plants.

"Total" and "soluble" phosphatase in leaves.—Sharma and Giri (2) have shown that the phosphatase occurs in two different forms in certain seeds. The one which can be extracted easily with water and therefore exists in "free" form is designated as "soluble phosphatase" and the other which cannot be extracted with water and therefore exists in "bound" form is designated as "bound phosphatase." Booth (4) has recently confirmed this concept of 'bound' and 'soluble' phosphatases in cereals. The occurrence of these two forms of phosphatase in leaves was investigated. In the following table are given the results of investigation. For purposes of comparison the phosphatase activity of cereals is included.

TABLE V.

'Total' and 'soluble' phosphatase in leaves.

Name of the leaves.	Phosphatase activity. Total	Soluble	"Soluble" per cent of "total".
Rice (Leaf)	7.5	7.9	100
Cholam	9.13	9.13	100
Ragi	11.88	11.88	100
French bean	51.10	51.10	100
Papaya	16.75	8.25	50
Akanda	11.78	4.23	36
Rice (Grain)	2.55	0.68	27

The results show that in some leaves like papaya, snake gourd the phosphatase exists partly in bound form while in others like rice, ragi, cholam, entirely in soluble form. It is rather difficult to explain why the phosphatase exists in certain leaves in completely soluble form and in others partly soluble and partly bound form. It may be stated here that drying of the leaves does not alter the solubility of enzymes as it was found that the activity of the enzyme obtained from fresh and dried papaya leaf material after extraction and filtration is practically the same in both the cases.

Variation in phosphatase activity with the age of leaf and fruit.—The various constituents of the leaf is generally influenced by the age of the leaf and the plant. It is, therefore, necessary to know the variation in phosphatase activity with the age of the leaves for studying the rôle of the enzyme in plant growth and metabolism.

Leaves of varying age from the same plant were collected and dried as described before. The total phosphatase activity of the different samples was determined. The plants investigated were papaya, french beans, snake gourd and akanda (*calatropis gigantea*). The results are presented in Table VI.

TABLE VI.

Variation in phosphatase activity of leaves and fruits with age.

Name of the plant.	Age and type (different stages in growth)	Phosphatase activity.
Papaya (*carica papaya*)	(a) 1st leaf (very tender)	13.7
	(b) 2nd leaf (tender)	18.25
	(c) 3rd leaf (fairly tender)	23.53
	(d) Medium sized leaf	17.45
	(e) Very old leaf	10.2
	(f) Dry leaf	8.9
Snake gourd	(a) Tender leaf	23.78
	(b) Old leaf	9.25
Akanda (*calotropis gigantea*)	(a) Tender leaf	16.95
	(b) Old leaf	11.78
French bean	(a) Young leaf	48.55
	(b) Medium sized leaf	51.1
	(c) Old leaf	27.4

The results show that the phosphatase activity decreases gradually with the age of the leaf. This relationship between the phosphatase activity and the age of the leaves was found to hold good in the case of the leaves of all plants investigated, namely, papaya, snake gourd, ȧkanda (*calotropis gigantea*) and french bean.

Distribution of phosphatase in the leaf.—In all the experiments described in the present paper the whole leaf was used for the determination of the activity of the enzyme. It was however thought interesting to know whether the various parts of the leaf possess the same activity. The phosphatase activity was therefore determined in various parts of the papaya and french bean leaves. The various parts of the leaf namely the (*a*) outermost edge, (*b*) middle strip, (*c*) inner strip, and (*d*) the midrib as indicated in figures 1 and 2 were carefully removed and dried. The phosphatase activity of the various samples was determined and the results are presented in Table VII.

TABLE VII.

Distribution of phosphatase in the leaf.

Name of leaf	Portions of the leaf	Phosphatase activity.
French bean	a) Outermost edge (5 mm. width)	32.1
	b) Middle strip (10 mm. width)	31.8
	c) Inner strip (10 mm. width)	31.8
	d) Midribs	15.4
Papaya	a) Outermost edge	21.25
	b) Middle strip	24.08
	c) Inner strip	21.12
	d) Midribs	12.375

The results show that the enzyme is uniformly distributed throughout the lamina of the leaf. The midrib however contains less phosphatase (about 50%) than the other parts of the leaf.

Leaf phosphatase activity and hydrogen ion concentration.—The optimum *p*H of the activity of plant phosphatase is reported by various workers to be 5.0-5.5. In the case of leaf phosphatase, Ignatieff and Wasteneys (1) have reported the *p*H5.5-5.9 as optimum for the activity of Canada Wonder bean. This is rather too high for plant phosphatases.

The following experiments were carried out with a view to determining the optimum *p*H for the phosphatase activity of papaya leaves. The determination was made both by colorimetric and potentiometric methods. Leaf powder was used as such for the determination of activity.

The results are presented in Table VIII.

TABLE VIII.

Leaf phosphatase activity and hydrogen ion concentration.

*p*H	3.6	4.0	4.4	4.8	5.2	5.6
Activity	9.4	11.77	17.75	20.55	21.7	17.5

The results show that the optimum *p*H for activity of the enzyme lies at *p*H 5.2 for papaya leaves.

Time of course of the action of leaf phosphatase.

The reaction mixture contained 20 c.c of M/5 acetic acid acetate buffer (*p*H 5.2), 20 cc. of 1% sodium β-glycerophosphate, 10 cc. of enzyme solution (prepared by extraction of french bean leaf powder with water in the proportion 1:10). The reaction was allowed to proceed at 35° and at stated intervals of time an aliquot of the reaction mixture was taken for the determination of inorganic phosphorus. In the following table are presented the results obtained on the increase in inorganic phosphorus formed in the reaction mixture with the time of incubation.

TABLE IX.

Time course of leaf phosphatase activity.

Time of hydrolysis in minute.	Inorganic phosphorus formed in the reaction mixture.	Ratio : : Time of hydrolysis / Ino. P. in mg.	Hydrolysis.
5	1.43 mg.	3.4	14.1
10	3.17	3.17	31.3
15	4.62	3.24	45.7
20	6.08	3.28	60.1
30	7.43	4.01	73.5
40	9.00	4.44	90.0
50	10.18	4.91	100.0
60	11.58	5.18	114.0

The results show that the rate of formation of inorganic phosphorus is strictly proportional to the time of hydrolysis until 60 per cent of the substrate is hydrolysed. After 60 per cent hydrolysis the rate of hydrolysis decreases. For the determination of the activity of leaf phosphatase it is, therefore, necessary to restrict the observation within the 60 per cent hydrolysis limit.

Relation between substrate concentration and leaf phosphatase activity. In Table X are presented the results obtained on the relation between phosphatase activity and the substrate concentration. The substrate solution was always adjusted to *p*H 5.2 by neutralising with acetic acid and then used for determining the activity by using 0.2 g. of dry leaf-powder. The total volume of the reaction mixture was 25 cc.

TABLE X.

Influence of substration concentration on phosphatase activity.

Substrate concentration	Phosphatase activity.	
	Papaya leaf	French bean leaf
0.4%	19.4	30.1
1.0	33.33	...
2.0	47.18	88.00
3.0	55.55	104.87
4.0	61.00	112.50
5.0	67.55	126.87
6.0	78.13	134.25
7.0	78.13	138.00
8.0	78.13	...
10.0	78.13	...

The results show that the substrate concentration for maximum activity is 6.0 per cent.

Discussion

The present data show the wide distribution of phosphatase and its occurrence in high concentration in the leaves of plants compared to other plant materials, such as cereals, tubers, etc. The phosphatase exists in them mostly in soluble form. In the case of some leaves like papaya, snake gourd etc., the enzyme exists in partly "bound form" which is difficult to extract with water.

In the preparation of leaf material used for activity determinations the need for the selection of a suitable method of drying the leaves without destroying the enzyme is emphasised and a simple method for preparing leaf material has been suggested.

The present study enables us to show that there is a relationship between the phosphatase activity of leaves and fruits and the growth and maturity of the plant. The data presented in Table VI would show the variation in phosphatase activity of the leaves of all the plants investigated, with the age of the leaves, the young leaves containing more phosphatase than the old ones. Thus the phosphatase activity is affected with the age of the leaf and its metabolic activity. These differences in composition of the leaves with varying age with respect to its phosphatase activity emphasises the necessity of sampling similar type leaves in any diagnostic work based on leaf analysis. The phosphatase is uniformly distributed in the lamina of the leaf.

An inspection of the results in Table VII shows that the lamina of the leaves is high in phosphatase activity while the midrib is considerably low (50 per cent of that of lamina without the midrib) in phosphatase. This highly significant difference in concentration of the enzyme in lamina and the midrib is an important observation which should be taken into consideration in studies on the variation in phosphatase activity of leaves with the metabolic activity of the plant.

In addition to the above, many factors appear to influence the phosphatase activity of leaves including soil, pathological conditions of the plant and others. It is not within the scope of this study to attempt to determine the individual effects of these factors. Such a study would require the growing of plants under controlled conditions. Our preliminary experiments on the influence of fertilizer treatment on phosphatase activity of leaves of papaya have however indicated certain significant variations in the enzyme with the type of fertiliser used. Much more work is still required before we can obtain a clear picture of the rôle of phosphatases in plant life.

SUMMARY

1. Phosphatase is widely distributed and occurs in high concentration in the leaves of all plants.

2. The enzyme occurs in certain leaves like those of ragi, rice, etc., in completely "soluble" form, while in others like papaya, snake gourd it exists in partly "bound" form which cannot be extracted with water.

3. The phosphatase activity is affected with the age of the leaf. The older leaves contain less phosphatase than the younger ones.

4. The lamina of the leaves is high in phosphatase activity while the midrib is very low in phosphatase. Leaf phosphatase exists its optimum activity at *p*H 5.2. The time course of the reaction of the leaf phosphatase is linear in character in the early stage of hydrolysis.

5. The rate of hydrolysis of the phosphatase varies with the substrate concentration until the concentration of the substrate is 6% and decreases further with increase in substrate concentration.

The author's thanks are due to Prof. V. Subrahmanyan for his interest in this investigation.

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OBSERVATIONS ON ASCORBIC ACID

PART I. STATE OF ASCORBIC ACID IN PLANT TISSUES.

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An increase in the indophenol titre of the extracts of certain vegetables on boiling was observed by Ahmad (1), and McHenry and Graham (2). This was interpreted to indicate the existence of combined ascorbic acid in such tissues. This view was not accepted by other investigators who ascribed the apparent increase wholly to the destruction of ascorbic acid oxidase present in such vegetables (Van Eekelen (3,4), Harris (5). Later in a series of studies Guha and his collaborators sought to obtain evidence of the existence of "ascorbigen" (combined ascorbic acid) in plant and animal tissues. (Guha and Pal 6, 7) ; Sen Gupta and Guha (8,9) ; Guha and Sen Gupta (10) ; Pal and Guha (11). These observations were confirmed by Levy (12), and Scarborough and Stewart (13, 14). Alcoholic, ethereal or chloroform extracts of dried cabbage ordinarily showing no reaction with the indophenol dye, even after passing of hydrogen sulphide in the cold, when hydrolysed with hot

water in an inert atmosphere, showed the presence of a reducing substance, the identity of which with ascorbic acid was established by the specific action of ascorbic acid oxidase and biological tests on scorbutic guinea-pigs. The amount of ascorbic acid in combined state in vegetable tissues was, however, small and several authors who failed to confirm these experiments believed this to be a normal variation falling within the range of experimental error (Mack 15) ; Mack and Tressler (16) ; Levy (17) ; Fugita and Ebihara (18, 19). In the mean time Readman and McHenry (20) advanced further proof of the presence of combined form of ascorbic acid in plants by chemical, spectrographic and biological assay and claimed this form to occur in combination with proteins, hydrolysing readily with 0.2% hydrochloric acid. The presence of combined ascorbic acid in urine was shown by Scarborough and Stewart (13); Banerjee (21) ; Banerjee, Sen and Guha (22, 23) ; Sen Gupta (24), and in blood by Saha, Majumdar and Guha (25). These latter authors based their proof of the presence of combined ascorbic acid on the fact that there is difference in the indophenol titre of the same extract after passing hydrogen sulphide in the cold and the hot condition. It is argued that any dehydroascorbic acid present would get reduced with hydrogen sulphide even in the cold. Therefore the increase in ascorbic acid value in the second treatment namely heating and then passing of hydrogen sulphide would represent combined ascorbic acid. More recently Harris and Olliver (26) have criticised the combined ascorbic acid hypothesis, stating that the supposed increase on cooking is due generally to a faulty technique and could be traced in most cases to either incomplete extraction, variation in sampling and the action of the oxidases. In their extensive studies these authors always obtained a decrease in ascorbic acid on cooking and the hydrolysis method of Reedman and McHenry (20) invariably gave a lower figure than the simple direct method recommended by these authors. They have not, however, explained the difference in value obtained by the action of hydrogen sulphide in the cold and the hot solution after acid extraction.

In this paper we have described the result of a critical study of this question.

Experimental

The methods used in this investigation for the estimation of free, dehydro and combined ascorbic acid are outlined below:—

Free Ascorbic Acid.—15 c.c. of 20% trichloracetic acid and 10 c.c. of 20% metaphosphoric acid were mixed together in a 25 c.c. stoppered glass cylinder. 10 gm. of fresh vegetable tissues were ground rapidly and thoroughly with approximately one-half of the above acid mixture and 5-10 gm. of pure white sand previously washed with hydrochloric acid. The whole was filtered rapidly through a muslin piece. The residue was returned to the mortar and extraction was repeated with the remaining acid mixture. Finally it was again filtered through the muslin piece. The whole extract was then centrifuged for 3-4 minutes and the volume made up to 100 c.c. It was then titrated with the standard solution of 2:6-dichlorophenol-indophenol and free ascorbic acid calculated,

Dehydro Ascorbic Acid.—20 c.c. of the aliquot were taken in a 200 c.c. flat bottomed flask. A moderate current of hydrogen sulphide was then passed through the extract for 15 minutes. The excess of gas was removed completely by passing a rapid current of carbon dioxide under pressure at room temperature. It was then titrated quickly against the standard indicator, and the value of free plus dehydro ascorbic acid was calculated. By deducting from the total the value of free ascorbic acid obtained in the first process, the value of dehydro ascorbic acid was obtained.

Combined Ascorbic Acid.—Again an aliquot of 20 c.c. was taken and a moderate current of hydrogen sulphide was passed for 15 minutes through the extract kept in a boiling water-bath. The excess of the gas was removed by a rapid current of carbon dioxide and titrated quickly against the indicator solution. From the difference in this titre and the value for free and dehydro-ascorbic acid the combined form of ascorbic acid was obtained.

The results obtained by the above procedure for a number of vegetables are shown in Table I.

TABLE I

State of Ascorbic Acid in vegetable tissues.

Name.	Ascorbic acid in mg./100 g. of fresh vegetable				
	Free.	Dehydro.	Combined.	Total.	Dehydro as % age of total.
Fenugreek (*Trigonella foenumgræcum*)	160.0	40.0	0.0	200.0	20.0
Turnip leaves (white) (*Brassica rapa*)	154.0	46.0	0.0	200.0	23.0
Bitter gourd (*Momordica charantia*)	133.0	27.0	0.0	160.0	16.9
Karem leaves	107.0	8.0	0.0	115.0	6.9
Turnip leaves (red) (*Brassica rapa*)	80.0	0.0	0.0	80.0	0.0
Cauliflower (*Brassica oleracea*)	80.0	20.0	0.0	100.0	20.0
Spinach (*Spinacia oleracea*)	75.5	1.5	0.0	77.0	1.9
Cabbage (*Brassica oleracea capitata*)	66.0	6.0	0.0	72.0	8.3
Mongra pods (*Raphanus Sativum*)	53.0	0.0	0.0	53.0	0.0
Mint (*Mentha viridis*)	50.0	11.5	0.0	61.5	18.7
Pea-pods (*Pisum sativum*)	40.0	0.0	0.0	40.0	0.0
Onion (*Allium cepa*)	40.0	0.0	0.0	40.0	0.0
Radish leaves (*Raphanus sativum*)	26.6	13.4	0.0	40.0	33.5
Peas (*Pisum sativum*)	26.0	0.0	0.0	26.0	0.0
Pumpkin (*Cucurbita maxima*)	26.6	5.4	0.0	32.0	16.9
Potatoe (*Solanum tuberosum*)	26.0	6.0	0.0	32.0	18.7
Onion leaves (*Allium cepa*)	15.0	5.4	0.0	20.4	26.4
Carrot (*Daucus carota*)	6.8	2.2	0.0	9.0	24.4
Lettuce	6.6	2.2	0.0	8.0	25.0

From Table I it is evident that most of the vegetables tested are rich sources of ascorbic acid when fresh, and those shown in first half of the table are even richer than citrus fruits, prominent among which are orange and lemon having an average value of 50 mg/100 gm. The amount of dehydro-ascorbic acid ranged from 0—33% of the total ascorbic acid present in different vegetables. It is possible, however, that some of free ascorbic acid is converted into dehydro form during the process of extraction through the action of the oxidase. Attempts were made to prevent such oxidation as far as possible, but the action of ascorbic acid oxidase is sometimes exceedingly rapid. However in some vegetable tissues the value of dehydro-ascorbic acid is quite high. No explanation can be given at this stage of the significance of dehydro-ascorbic acid present in different plant tissues.

No appreciable amount of combined ascorbic acid was detected in any vegetable tissue by this method. From the results of these experiments it can be safely cncluded that there was no increase in ascorbic acid content on heating. As Harris and Olliver (11) have suggested it is possible that three causes have combined to produce the misleading appearance of increase in ascorbic acid after heating, recorded by McHenry and others, and responsible for combined ascorbic acid hypothesis in plant tissues: (*a*) Incomplete extraction: From unheated tissues, particularly if they are of a hard and fibrous nature, quantitative extraction of ascorbic acid is more difficult than from heat-softened materials. Thus a fictiously low value is given to the raw product unless a satisfactory method of extraction is used. (*b*) Action of oxidase: The most important factor may be the ineffective procedure used to prevent the action of oxidases during the preliminary extraction of the raw material and the destruction of the same oxidases by the heating or cooking process. (*c*) Variation in sampling: This factor may also produce gross errors. A representative sampling of the vegetable is often difficult.

We next followed the method of Reedman and McHenry as modified by Harris and Olliver (11) which is outlined below for comparison. The material (10 gm.) was ground with sand in the presence of 50 c.c. of 0.2% HCl and transferred to a small wide mouthed flask. H_2S was passed into the solution for 10 minutes and the flask was stoppered and kept at 40° for one hour. The solution was then squeezed through muslin into a wide-mouthed flask. The residue was extracted with 12.5 c.c. of 20% trichloracetic acid and 5 c.c. of 20% metaphosphoric acid and washed into the flask, the volume being kept below 100 c.c. H_2S was passed through the solution for a further 5 minutes ; the flask was stoppered and left overnight. H_2S was removed in a stream of CO_2 and volume made upto 100 c.c. in a graduated flask. The solution was then filtered and titrated. This titration gives value of total ascorbic acid in the material i.e., the reduced reversibly oxidised and combined form of the acid.

Side by side usual method of extraction as outlined before was followed and free and dehydro ascorbic acid was determined. The results for three vegetables which have been reported to give a combined ascorbic acid value out of the large number of experiments are shown in Table II.

TABLE II

Name of vegetable.	*Ascorbic acid in mg. per 100 g. of fresh vegetables.	
	By method adopted by the authors.	By modified Reedman and McHenry method.
Cauliflower (*Brassica oleracea brytes*)	100.3	100.06
Mint (*Mentha Viridis*)	61.2	61.0
Fenugreek (*Trigonella Fœnum-græcum*)	200.0	198.8

*Average of 3 values.

It will be seen that there is no difference between the values and neither there is any decrease nor increase in the value of total ascorbic in the vegetables examined by the two methods. This result confirms the veiw that there is no combined ascorbic acid present in the vegetable tissues. It may be mentioned that Harris and Olliver (11) found a decrease in all the vegetables they examined which suggested oxidation to have taken place during their experiments. Our experiments however, did not show any appreciable lowering of the value.

Further investigation into the combined ascorbic acid hypothesis was carried out by the following two methods: (*i*) It was reported by Reedman and McHenry (18) that ascorbic acid occurs in combination with proteins in the vegetables. To examine this hypothesis the following procedure was adopted.

Pepsin was used to hydrolyse the protein complex. 0·5% solution of pepsin was prepared in 0.1-N HCl. 10 c.c. out of it were added to 20 c.c. of the water extract and the trichloracetic-metaphosphoric acid extract and incubated for 3 and 5 hours. The treatment would release the ascorbic acid, if present, bound with proteins, and could be estimated by titration. The results are shown in Table III and IV.

TABLE III

Name of vegetable.	Time of incubation.	pH.	Ascorbic acid mg./100 g. of fresh vegetable.			
			Water extract.		Trichloracetic-metaphosphoric Acid extract.	
			Control (without pepsin)	Test (with pepsin)	Control (without pepsin)	Test (with pepsin)
Karem leaves.						
	3 hours.	1.1	33.3	33.3	40.0	33.3
	5 hours.	,,	18.1	16.5	20.5	25.0
Fenugreek.						
	3 hours.	,,	40.0	40.0	40.2	45.2
	5 hours.	,,	32.5	27.8	33.4	30.0

TABLE IV

Name of vegetable.	Time of incubation (Hrs.) pH		Ascorbic acid mg./100 g. of original vegetable.					
			Water extract.		Acid mixture extract.		Residue after single water extraction.	
			Control (without pepsin).	Test (with pepsin).	Control (without pepsin).	Test (with pepsin).	Control (without pepsin).	Test (with pepsin).
Karem Sag.	1.1	3	61.8	62.4	85.4	75.0	10.50	9.45
	,,	5	53.35	51.2	70.5	66.0	10.50	9.02
Fenugreek.	,,	3	83.8	82.2	99.8	98.4	12.50	10.8
	,,	5	69.24	68.5	78.2	73.1	12.00	9.65
Cauliflower	,,	3	36.1	35.5	44.0	36.4	12.00	10.5
	,,	5	21.8	22.4	28.5	25.0	...	...

It will be seen that there is no increase in the ascorbic acid value after incubation with pepsin of the water extract, or acid extract or residue after water extraction of the vegetables examined. The incubation was carried for 3 and 5 hours. These experiments give no indication of the release of any combined ascorbic acid from its complex and hydrolysed by pepsin. Oxidation of extracts takes place due to rather long incubation.

(ii) In order to make the problem of bound ascorbic acid still more clear, the treatment of the vegetable extract was carried out with taka-diastase known to hydrolyse complex carbohydrates, thus releasing any organic compounds in combination with them. Water extract of vegetable was prepared and its *p*H *adjusted* to 5.28 which is the optimum *p*H of takadiastase. 10 c.c. of 1.0% takadiastase solution in water were added to 20 c.c. of the water extract and incubated for 2 hours. Two drops of toluene were added to avoid fermentation. After various intervals of time ascorbic acid was extracted with acid mixture and titrated against the dye.

TABLE V

Vegetable.	Time of incubation.	Ascorbic acid mg./100 gm.	
		Control (incubation without takadiastase)	Test (incubation with takadiastase)
Fenugreek.			
	30 Minutes.	175.0	175.0
	1 Hour.	116.0	115.4
	2 Hours.	77.7	77.0
Mint.			
	30 Minutes.	42.4	42.6
	1 Hour.	36.7	36.2
	2 Hours.	25.4	25.0

It is evident from Table V that there is no difference in value of the test and control which shows no indication of ascorbic acid present in combination wth carbohydrates. Oxidation takes place in spite of various precautions.

SUMMARY

(1) A critical examination of the literature regarding the state in which ascorbic acid exists in various plant tissues has been made.

(2) The amounts of free ascorbc acid, dehydroascorbic acid and combined ascorbic acid as assessed by different technique have been determined in some twenty vegetables. The amount of dehydroascorbic acid varies from 0-33% of the total amount present.

(3) In none of the vegetables examined has any combined ascorbic acid been detected. No increase in ascorbic acid value could be found by any of the techniques used by the earlier investigators or by incubation with pepsin or taka-diastase which would release ascorbic acid from complexes with proteins or carbohydrates.

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OBSERVATIONS ON ASCORBIC ACID
PART II. STATE OF ASCORBIC ACID IN URINE

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Early work on vitamin C failed to detect any vitamin in urine (1). Later Van Eekelen (2) and Van Eekelen *et al* (3) mentioned without actual data that the reducing substances in urine are greater in persons consuming much fruit or higher doses of decitrated lemon juice. They also stated that this capacity of reduction gradually disappears from urine of guinea-pigs on a scorbutic diet. Harris, Ray and Ward (4) definitely proved the excretion of vitamin C in urine, and on the level of vitamin C in the urine based a method for the assessment of vitamin C sufficiency in the diet. They observed that normal individuals showed a surprisingly constant indophenol reducing capacity, the daily output being about 30-33 mg. Johnson and Zilva (5) confirmed this observation and showed that two factors were involved in the excretion of ascorbic acid in the urine of normal individuals, (i) the degree of saturation of the subject, and (ii) the magnitude of intake, and further proved that the reducing capacity of urine was definitely due to ascorbic acid. Abbasy, Harris, Ray and Marrack (6) discussed the specificity of the indophenol method for vitamin C estimation in urine in view of the statement of Van Eekelen (2) that it may also indicate traces of other substances present in urine e.g., thiosulphate etc. They observed however, that in practice, these substances were small and insignificant and hence the method was quite specific and adequate for clinical purposes. Ahmad (7) studied the reducing capacity of human urine excreted under different dietary conditions and found considerable increase in reducing action of urine on high meat diets without increasing the vitamin C intake and showed that the major part of reducing action was due to ascorbic acid. Scarborough and Stewart (8) described a method for the estimation of vitamin C in urine in the presence of interfering substances using mercuric acetate.

Sen Gupta and Guha (9), and Bannerji, Sen and Guha (10, 11) indicated the existence of "ascorbigen" *i.e.*, combined ascorbic acid in human urine, the quantity

increasing under certain conditions. In the present investigation a number of samples of urine have been examined from normal persons as well as those suffering from diseases. The observations of Guha and his colleagues have been generally confirmed.

EXPERIMENTAL

Generally 24 hours but in some instances three hour samples of urine were collected from 10 A.M. to 1 P.M. in presence of 10% acetic acid. Banerji (12) has shown that 5% of sulphuric acid is the best preservative, but it was found that it charred the organic matter of urine and rendered difficult the subsequent titration against the dye. The urine was kept in dark-coloured bottles, until titrated which was undertaken within a few hours of the collection of the sample.

The aliquots of the collected urine were examined for

(i) Free ascorbic acid,

(ii) Dehydro-ascorbic acid, and

(iii) Combined form of ascorbic acid.

The method used for the estimation of the three states of ascorbic acid has been fully discussed in an earlier communication (Ahmad, Qureshi, and Babbar, 13). The results of the examination of urine sample obtained from a large number of normal individuals and those suffering from various diseases are recorded in Tables I to V.

TABLE I

Excretion of Ascorbic Acid in Urine of persons in normal health in 3 hours.

No.	Volume of total urine (ml.)	Ascorbic acid in total volume of urine.			
		Free (mg.)	Dehydro- (mg.)	Combined (mg.)	Total (mg.)
1.	200	2.80	0.20	0.0	3.00
2.	155	3.10	0.93	0.0	1.03
3.	260	10.10	0.00	0.0	10.10
4.	371	1.80	0.00	0.0	1.80
5.	263	2.10	0.52	0.0	2.62
6.	280	5.60	0.00	0.0	5.60
7.	100	2.00	0.00	0.0	2.00
8.	130	1.30	0.60	0.0	1.90
9.	150	6.00	0.26	0.0	6.26
10.	180	2.31	2.00	0.0	4.31
11.	65	1.01	0.40	0.0	1.41
12.	100	1.20	0.08	0.0	1.28
13.	170	2.21	0.10	0.0	2.31
14.	175	3.50	0.91	0.0	4.41
15.	70	0.80	0.50	0.0	1.30
16.	243	4.86	1.61	0.0	6.50
17.	120	1.20	0.00	0.0	1.20
	Average	3.25	0.48	0.0	3.73

TABLE II

Excretion of Ascorbic Acid in the urine of average normal individuals in 24 hours.

No.	Age Yrs.	Volume of total urine (ml).	Ascorbic acid in. Free state. (mg.)	Dehydro state. (mg.)	Combined state. (mg.)	Total. (mg.)	Combined as % of total.
1.	25	886	70.3	11.7	16.4	98.4	16.6
2.	25	1550	70.3	11.5	8.9	90.4	9.8
3.	37	1810	64.0	12.3	10.7	87.0	12.3
4.	36	1110	63.0	3.4	7.9	74.3	10.6
5.	38	1115	59.0	6.7	6.8	72.8	9.4
6.	28	1230	56.0	7.4	8.7	72.1	12.07
7.	35	476	56.5	10.4	4.3	71.2	6.04
8.	25	968	60.1	5.4	3.4	68.8	4.9
9.	25	1020	48.5	11.5	8.3	68.3	12.2
10.	36	1190	53.6	11.2	3.4	68.0	5.0
11.	25	1172	47.4	13.1	6.5	67.0	9.7
12.	31	1210	52.0	6.7	7.5	66.2	11.3
13.	35	980	57.0	1.7	1.3	62.7	2.1
14.	22	1015	50.9	4.3	6.7	61.9	10.8
15.	26	870	40.3	5.7	14.4	60.4	23.9
16.	25	890	43.9	12.9	3.4	60.2	5.6
17.	21	910	48.0	5.3	6.7	60.0	11.2
18.	31	1230	41.6	10.6	5.3	57.5	9.2
19.	30	1100	46.0	7.0	4.2	57.2	7.3
20.	36	890	49.0	3.4	4.3	56.7	7.5
21.	16	810	40.3	8.7	7.6	56.6	13.4
22.	35	752	43.8	6.14	6.5	56.1	11.5
23.	35	1451	44.8	2.7	8.7	56.2	15.4
24.	36	700	48.7	3.1	3.2	55.0	5.8
25.	30	810	34.8	10.2	10.0	55.0	18.1
26.	16	930	42.4	7.6	4.3	54.3	7.9
27.	10	770	36.4	10.2	6.5	53.1	12.2
28.	27	610	41.4	8.4	2.3	52.1	4.4
29.	37	650	36.4	10.5	4.6	51.5	8.7
30.	20	980	38.6	8.3	3.5	50.4	6.9
31.	18	770	38.5	8.4	3.5	50.4	6.9
32.	41	750	37.8	8.8	3.6	50.2	7.1
33.	14	720	36.3	4.5	9.2	50.0	18.4
34.	16	950	40.0	3.2	5.4	48.6	11.1
35.	50	670	36.0	6.7	5.6	48.3	11.6
36.	12	730	40.5	3.5	3.7	47.7	7.7
37.	43	810	36.3	3.6	6.5	46.4	13.9
38.	21	1160	40.3	2.7	3.0	46.0	6.5
39.	40	800	28.0	10.0	7.2	45.2	15.9
40.	45	1112	38.0	3.8	3.4	45.2	7.5
41.	36	750	32.6	5.8	6.2	44.6	13.9
42.	35	820	35.4	1.4	7.0	43.8	15.9
43.	25	770	38.8	1.2	2.8	42.8	6.5
44.	40	810	36.0	0.0	6.7	42.7	15.7
45.	12	1200	26.8	6.8	8.6	42.2	20.3
Average value			45.2	6.7	6.2	58.1	10.68

TABLE III

Daily excretion of Ascorbic Acid in the urine of persons having no organic disease but having poor nutrition.

No.	Age	Total volume of urine. (ml).	Ascorbic acid in. Free state. (mg.)	Dehydro state. (mg.)	Combined state. (mg.)	Total. (mg.)	Combined as % of total.
1.	28	630	31.0	6.5	10.5	48.0	21.9
2.	31	718	30.5	10.2	6.5	47.0	30.8
3.	40	1170	26.0	7.2	7.4	40.6	18.2
4.	18	440	25.8	7.2	7.3	40.3	18.3
5.	12	741	25.0	7.5	6.4	38.9	16.4
6.	30	600	23.4	1.2	10.4	35.0	29.7
7.	25	760	24.4	0.0	9.9	34.0	28.9
8.	26	600	20.2	6.5	7.1	33.6	21.1
9.	30	1140	26.4	1.1	5.8	33.3	17.4
10.	50	1120	15.0	8.0	10.0	33.0	30.3
11.	38	450	20.0	5.0	3.0	28.0	10.7
12.	30	350	15.0	2.0	2.5	19.5	12.8
13.	70	230	14.3	0.8	3.4	18.5	18.4
14.	11	1000	7.25	1.5	4.99	13.7	36.4
Average value			21.7	4.6	6.8	33.1	20.51

TABLE IV

Excretion of Ascorbic Acid in the urine of persons suffering from various pathological conditions in 24 hours.

Patient No.	Age (yrs.)	Total volume of urine (ml.)	Ascorbic acid in Free state. (mg.)	Dehydro state. (mg.)	Combined state. (mg.)	Total. (mg.)	Combined as % of total.	Disease.
(a) 1.	25	1320	12.2	13.8	28.6	54.6	52.4	Cough, cold and bronchitis.
2.	21	650	22.0	15.0	12.5	49.5	25.3	,,
3.	26	560	27.0	3.8	10.6	41.4	25.6	,,
4.	18	908	26.5	6.7	4.7	37.9	12.4	,,
5.	30	961	25.0	5.9	3.7	34.6	10.7	,,
6.	36	950	23.6	2.0	6.0	31.6	19.0	,,
Average value			22.7	7.9	11.0	41.6	26.4	
(b) 1.	30	1310	29.0	11.4	17.4	57.8	30.1	Malarial fever.
2.	35	561	32.0	5.3	14.7	52.0	28.3	,,
3.	32	1490	30.0	8.3	9.5	47.8	19.9	,,
4.	27	640	30.0	2.3	8.5	40.8	20.8	,,
5.	40	450	27.0	2.2	11.0	40.2	27.4	,,
6.	25	660	30.0	3.6	4.5	38.1	11.8	,,
7.	36	350	16.0	0.2	1.0	17.2	6.0	,,
Average value			27.7	4.8	9.5	42.0	22.6	
(c) 1.	15	450	50.0	37.8	25.0	112.8	22.2	Dermatitis.
2.	21	890	30.0	16.1	14.0	60.1	23.1	,,
3.	25	1380	14.1	9.1	12.8	36.0	35.5	,,
Average value			31.4	21.1	17.2	69.7	21.9	
								Miscellaneous.
(d) 1.	20	2430	51.9	4.5	53.4	109.8	48.5	Diabetes.
2.	36	400	18.5	18.3	18.7	55.5	33.7	Intestinal colic.
3.	35	330	20.8	4.9	8.3	30.0	27.7	,,
4.	38	450	30.1	3.9	6.8	40.8	16.6	Colitis.
5.	43	613	28.0	12.4	14.5	54.9	33.3	Dropsy.
6.	32	1200	17.7	13.8	10.5	42.0	25.0	Renal colic.
7.	28	756	30.2	12.0	10.4	52.6	19.9	Hepatitis.
8.	38	1330	30.3	2.4	10.5	43.2	24.3	Cellulitis.
9.	36	570	18.1	14.6	1.9	34.6	5.47	Paraplegia.
10.	50	750	35.7	5.9	8.4	50.0	16.8	Monoplegia.
11.	45	300	17.2	1.7	4.9	23.8	20.5	Ascites.
12.	60	600	11.4	1.8	1.8	15.0	11.9	Sciatica.
13.	35	330	20.8	0.9	8.3	30.0	27.7	Colitis.
14.	36	530	16.1	2.3	3.3	21.7	15.2	Gonococcal infection and pain in joints.
Average value			21.8	6.8	11.55	43.15	26.8	

TABLE V

Excretion of Ascorbic Acid in the urine of persons suffering from pulmonary tuberculosis in 24 hours.

No.	Volume of total urine. (ml.)	Ascorbic acid in mg./total volume of urine in the			
		Free and dehydro state. (mg.)	Combined state. (mg.)	Total. (mg.)	Combined as % age of total.
1.	1500	30.00	30.00	60.00	50.00
2.	610	10.62	8.96	19.58	42.20
3.	400	20.00	20.00	40.00	50.00
4.	1100	11.00	2.75	13.75	20.00
5.	1000	10.00	10.00	50.00	20.00
6.	1360	9.16	6.81	16.00	42.70
7.	1000	10.00	10.00	50.00	20.00
8.	2100	69.60	60.00	129.60	46.30
9.	1960	49.00	19.00	98.00	50.00
10.	1100	13.20	5.50	18.70	29.40
11.	1080	18.36	12.53	30.89	40.60
12.	1260	36.51	21.46	61.00	40.10
13.	800	26.10	13.60	10.00	31.00
14.	820	8.20	8.20	16.40	50.00
15.	1160	11.60	11.60	23.20	50.00
16.	1900	76.00	19.00	95.00	20.00
17.	700	3.50	4.90	8.40	58.30
18.	610	42.20	37.80	80.00	47.30
19.	710	37.30	21.20	61.50	39.30
20.	130	33.30	20.70	51.00	38.30
21.	810	28.90	21.70	53.00	46.60
22.	900	32.80	20.70	53.50	38.60
23.	970	32.60	18.90	51.50	36.60
24.	1700	28.30	20.20	48.50	41.60
25.	1150	25.00	23.00	48.00	47.90
26.	1100	30.30	16.70	47.00	35.50
27.	1000	24.10	22.10	46.00	48.10
28.	510	29.20	15.30	11.50	31.50
29.	1650	25.35	18.00	43.35	41.50
30.	870	28.00	16.00	42.20	37.40
31.	2000	25.10	16.80	41.90	40.10
32.	600	21.00	16.00	40.00	41.10
33.	1315	19.40	15.60	35.00	37.20
34.	1300	20.50	12.00	32.20	41.50
Average value		28.20	18.70	46.90	39.90

Discussion

The urine of 140 persons has been examined for free, dehydro, combined and total ascorbic acid. The data can be arranged in 8 groups, as shown in Table VI.

If the first group which includes persons in normal health, the average total excretion was 29·84 mg. which is the lowest average figure when all the groups are taken into consideration. This group includes persons in which no combined ascorbic acid has been detected. It may be pointed out, however, that only 3 hours urine (10 A.M.—1 P.M.) of the persons in this group was examined. It is possible that multiplying these figures by 8 does not represent the true daily output of ascorbic acid, and in those hours of the day no appreciable amount of combined ascorbic acid is excreted.

The second group includes 45 persons in average normal health and of different ages (Table II). These investigations were undertaken in the months of June and July which is the mango season when rather large amount of mangoes are consumed. The total output of ascorbic acid in this group was between 42 and 98 mg. (average 58 mg.), of which 78.3% was found to be in the free state, 11.3% as dehydro ascorbic acid, and 10.4% in the combined state. In the next category (Table III) 14 persons with poor nutrition were grouped. They showed an average total excretion of 33 mg. of which 67.1% was free, 13.0% dehydro and 19.4% combined ascorbic acid.

Next four categories constitute groups of persons with respiratory diseases, malarial fever, dermatitis and miscellaneous pathological conditions. Their total average excretion of ascorbic acid varied from 28.0—43.2 mg. of which 15.3—26.0% was found to be in the combined state.

The last group consists of 34 patients suffering from pulmonary tuberculosis (Table V). These were cases from a local hospital. Their average total daily output worked out to be 46.9 mg. of which as much as 39.7% was in the combined state. The diet of these patients was rich in fruits and vegetables and hence the rather high output of total ascorbic acid.

TABLE VI.

Average daily output of Ascorbic Acid (mg.) by groups of persons in health and disease.

Physiologic condition.	Free ascorbic acid		Dehydro ascorbic acid		Combined ascorbic acid		Average total excretion	Average % age of free ascorbic acid.	Average % age of dehydro ascorbic acid.	Average % age of combined ascorbic acid.
	Range	Average value	Range	Average value	Range	Average value				
Persons in normal health.	8.32-83.2	26.0	0.0-16.0	3.84	0.0	0.0	29.81*	87.13	12.87	0.0
Persons with average normal health.	26.8 -70.3	15.1	0.0-13.1	6.7	1.3-16.4	6.2	58.13	77.7	11.62	10.68
Persons with poor nutrition but no organic disease.	7.25-31.0	21.7	0.0-10.2	4.6	2.5-10.5	6.8	33.1	65.56	13.90	20.54
Persons with respiratory diseases.	12.2 -27.0	22.7	2.0-15.0	7.9	3.7-28.6	11.0	41.6	51.6	19.00	26.4
Persons with miscellaneous pathologic conditions.	11.1 -51.9	21.8	0.9-18.3	6.8	1.8-53.1	11.55	43.15	57.5	15.7	26.8
Persons with malarial fever.	16.0 -32.0	27.7	0.2-11.4	4.8	1.0-17.4	9.5	42.0	65.95	11.15	22.6
Persons with Dermatitis.	14.1 -50.0	31.4	9.1-37.8	21.1	12.8-25.0	17.2	69.7	44.9	30.2	21.9
Persons with pulmonary tuberculosis.	3.5 -76.0 (Free + dehydro)	28.2 (Free + dehydro)	...	...	2.75-60.0	18.7	46.9	60.1 (Free + dehydro)	...	39.9

*During three hours from 10 A.M. to 1 P.M.

It would be noticed that generally speaking all the individuals examined had rather high total output of ascorbic acid in the urine. This would indicate that almost all the persons were in a good state of nutrition with respect to vitamin C. It is doubtful whether any significance can be attached to the amount of dehydro-ascorbic acid present in the samples of urine examined. It may only represent an oxidation which takes place during the interval between the time of excretion and examination of the urines.

The amount of combined ascorbic acid found in the urine of different groups of individuals is rather striking. The first group of persons in normal health showed none at all. The second group of persons in average normal health showed an average of 10.4% combined ascorbic acid. Again persons in poor nutrition, or having mild forms of infections such as respiratory disease, dermatitis, or miscellaneous pathological conditions and even malarial fever had as much as 15—26% of the urinary ascorbic acid in the combined form. In an acute and heavy infection such as that of pulmonary tuberculosis the percentage of combined ascorbic acid excreted in the urine was high, generally between 30 and 50% (average 39.7%). It is significant and supports the view of Bannerji, Sen and Guha (9) that vitamin C may act as a detoxicating agent combining with the toxins in the body.

SUMMARY

The daily excretion of ascorbic acid in the urine of 140 persons of different ages and in different conditions of health and disease has been estimated. The ascorbic acid was estimated as existing in free, dehydro and combined forms.

The total output of ascorbic acid in all the individuals examined was somewhat high indicating a satisfactory state of nutrition with respect to this vitamin. This may be due to the fact that the observations were made during the season when mangoes are plentiful and cheap and find their way into the dietary of individuals at all economic levels.

Generally speaking 60—90% of the ascorbic acid excreted is in the free state, 10—50% in dehydro form and 10—40% in the combined state. No significance can be attached to the dehydro form, but the value of combined ascorbic acid increases in pathological conditions. It is quite high in the case of patients suffering from pulmonary tuberculosis which may be taken to support the view of Guha and his colleagues that ascorbic acid may act as a detoxicating agent.

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OBSERVATIONS ON ASCORBIC ACID
PART III: EFFECT OF METHODS OF PRESERVATION ON ASCORBIC ACID CONTENT OF FRUITS.

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(Received for publication July 4, 1945)

Considerable work has been undertaken in other countries on the effect on ascorbic acid content of processed fruits notable among which is that of Mamie Olliver (1). The present war has given great stimulus to the canning industry in this country. These rapid developments, however, in the absence of competition and the stress of war, have led to the employment of processes which are by no means conducive to the retention of the maximum nutritive value of the fruit. Through arrangement with a local firm, we have examined a number of preserved fruits, when freshly canned and after storing for a certain period. The results are reported in the Table I. The usual technique of assay was employed, the dehydro-ascorbic acid present having been reduced with hydrogen sulphide.

TABLE I

Name of Foodstuff.	Ascorbic acid in mg per 100 g						
	Fresh fruit.	Freshly canned.	after 7 days	15 days	30 days	60 days	90 days
Mango chutney	36.2	27.0	25.6	21.7	21.5	21.1	22.9
Tomato chutney	41.8	28.5	28.0	27.6	27.5	27.3	27.0
Mango pickle	61.5	47.2	42.4	36.6	33.5	30.7	31.0
Lemon pickle	56.7	52.0	41.5	36.8	31.1	33.6	32.0

The data show that there was considerable loss of ascorbic acid in the process employed for preservation. The loss is highest in the case of tomato being as much as 32% and least in the case of lemon about 8%. In the mango there was a slightly greater loss in the chutney (25.4%) than in the pickle (23.2%). After this initial loss there is a slow but progressive deterioration during storage in the first fortnight after which the rate of loss seems to be still smaller. Total loss of ascorbic acid during three months' storage was only about 5.2% in the case of tomato chutney, 15.2% in mango chutney, 34.3% in mango pickle, and 38.4% in the case of lemon pickle.

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ERRATA

Vol. IV, No. 1, Page 1
For "Received for publication, December 27, 1944."
read "Received for publication, December 27, 1943."

Vol. IV, No. 2, 1944, Page 57
For "The Stability and Absorption of the Antigens of Bact. typhosum with Special Reference to Oral Immunization."
read "The Stability and Absorption of the Antigens of Bact. typhosum with Special Reference to Oral Immunization. Part II."

Vol. IV, No. 2, 1944, Page 67
For "Vitamin C and Carbohydrate Metabolism. Part V."
read "Vitamin C and Carbohydrate Metabolism. Part VI."

Vol. IV, No. 2, 1944, Pages 82 and 83
For "butylcholine bromide and B.C.B."
read "butyrylcholine bromide."

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ERRATA

Vol. IV, No. 2, 1944, Pages 82 and 83

For "butylcholine bromide and B.C.B."

read "butyrylcholine bromide".

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NEW HÆMOSTATIC AGENTS. PART II. RELATION BETWEEN CHEMICAL CONSTITUTION AND HÆMOSTATIC ACTIVITY AS OBSERVED *IN VITRO*

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(Received for publication, April 10, 1944)

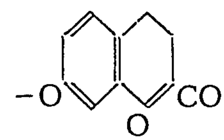
In Part I, an account of the hæmostatic activity of ayapanin (=herniarin) and ayapin, two natural coumarins isolated from *Eupatorium Ayapana*, Vent., has been given (1). The present communication describes the relationship between hæmostatic action, as observed *in vitro*, and chemical constitution of some compounds which are structurally related to these two compounds.

The clotting time of blood has been determined by the method followed previously. The hæmostatic activity (=H.A.) has been calculated in accordance with the formula given in Part I. Hæmostatic activity of the order of ±8 and less has been omitted from the tables, as such values have hardly any significance. Results obtained with various compounds are shown in the following tables.

TABLE I.
Coumarin, umbelliferone and O-substituted Umbelliferones.

Name of compound.	Normal clotting time (*n*): Min.-Secs.	Observed clotting time (*o*): Min.-Secs.	H. A. (Hæmostatic activity).
Coumarin	1-45	1-44	...
Umbelliferone	1-45	1-40	...
7-Methyl-umbelliferone (ayapanin)	1-45	0-56	47
7-Ethyl- ,,	1-50	1-7	39
7-*n*-Propyl- ,,	1-50	1-20	27
7-*iso*Propyl- ,,	1-45	1-21	23
7-Allyl- ,,	1-50	1-30	18
7-*n*-Butyl- ,,	1-45	1-48	...
7-*iso*Butyl- ,,	1-45	1-32	12
7-Benzyl- ,,	1-45	1-24	19
7-Ethylenebis- ,, (I)	2-00	2-10	...
7-Trimethylenebis- ,, (II)	2-00	1-56	...
Umbelliferone-7- acetic acid (III)	2-00	2-10	...
Acetylumbelliferone (IV)	1-50	1-45	...

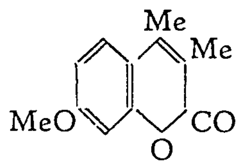
From the above data it is evident that the coumarin nucleus itself does not induce the physiological action. The introduction of an $-OH$ in position 7 does not make any difference. Alkylation of the phenolic group however brings about a profound change. It is moreover evident that the hæmostatic activity is maximum in the case of methyl umbelliferone, but diminishes, as a rule, with increase in the weight of the alkyl group. The isomeric propyl derivatives possess almost identical activity, but the introduction of a double bond in the alkyl chain (as in the allyl compound) causes the activity to be diminished. Between the two butyl compounds, the *iso*-compound has a slight activity, whereas the normal one is inactive. Although the benzyl group is much heavier than allyl, the activity is found to be of the same order. The compounds (I, II, III and IV) are without activity showing that mere etherification or esterification of the phenolic group cannot be responsible for the development of hæmostatic properties.

$R-(CH_2)_2-R$ (I) where R = 

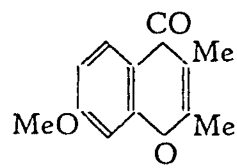
$R-(CH_2)_3-R$ (II)

$R-CH_2.CO_2H$ (III)

$R-CO.CH_3$ (IV)



(V)



(VI)

TABLE II.

7-Alkoxy-4-methylcoumarins.

Name of compound.	Normal clotting time (*n*): Min.-Secs.	Observed clotting time (*o*): Min.-Secs.	H. A.
7-Methoxy-4-methylcoumarin	1-48	1-1	44
7-Ethoxy- ,,	2-00	1-53	...
7-*n*-Propoxy- ,,	2-00	1-50	...
7-*iso*Propoxy- ,,	2-00	2-00	...
7-*n*-Butoxy- ,,	2-00	1-48	10
7-*iso*Butoxy- ,,	1-55	2-00	...
7-Methoxy-6-chloro-4-methylcoumarin	2-15	2-5	...

From the above table it will be seen that 7-methoxy-4-methyl-coumarin possesses a strong hæmostatic action which is comparable to that of 7-methoxy-coumarin. The introduction of a chlorine atom in position 6 completely suppresses the action. The replacement of the methyl group by other alkyl groups also completely stops the activity except in the case of the *n*-butyl compound which has a feeble action.

TABLE III.

7-Methoxydialkyl-coumarins and two chromones.

Name of compound.	Normal clotting time (*n*): Min.-Secs.	Observed clotting time (*o*): Min.-Secs.	H. A.
7-Methoxy-3:4-dimethyl-coumarin (V)	1-35	1-37	...
7-Ethoxy- ,,	1-40	1-40	...
7-Methoxy-3-ethyl-4-methylcoumarin	1-40	1-40	...
7-Methoxy-3-benzyl-4-methylcoumarin	2-15	1-58	13
7-Methoxy-2:3-dimethylchromone (VI)	1-37	1-22	16
7-Methoxy-2-methyl-3-styrylchromone	2-10	2-00	...

These data indicate that the hæmostatic activity of 7-methoxy-4-methyl-coumarin is much reduced or suppressed by the introduction of alkyl substituents in position 3. The chromone (VI), which is isomeric with the coumarin (V), shows definite, though feeble, activity. It is therefore probable that heterocyclic compounds other than coumarins may also share this interesting physiological property.

TABLE IV.

Dihydroxycoumarins and some of their derivatives.

Name of compound.	Normal clotting time (*n*): Min.-Secs.	Observed clotting time (*o*): Min.-Secs.	H. A.
Esculetin	1-42	1-39	...
Daphnetin	1-42	1-40	...
Scopoletin	1-30	1-15	17
Scopolin	1-50	1-47	...
Esculetin dimethyl ether	1-50	1-26	22
,, methylene ether (ayapin)	1-48	0-57	47
Daphnetin dimethyl ether	1-30	1-00	33
Dihydroayapin	2-00	1-58	...

Esculetin and daphnetin, like umbelliferone, are inactive. Activity appears after methylation of the –OH in position 6, but this feeble activity is suppressed by the introduction of a glucose residue as in scopolin. Solubility in water has no relation with the hæmostatic activity, because scopolin is easily soluble in water. Etherification of the –OH group of scopoletin increases the activity, as is to be expected, but the degree of increase is less than that observed in the case of the methylene ether, ayapin. Daphnetin dimethyl ether is more active than esculetin dimethyl ether showing that the –OMe group in position 8 is more powerful than that in position 6. The activity completely vanishes on reduction of the pyrone double bond as in 3:4-dihydroayapin.

In this connection it was thought desirable to study some natural coumarins of a more complex nature. The results are given in Table V.

TABLE V.

Some Natural Coumarins.

Name of compound.	Normal clotting time (n): Min.-Secs.	Observed clotting time (o): Min.-Secs.	H. A.
Angelecin	1-38	1-35	...
Psoralen	1-38	1-36	...
Bergapten	1-25	1-32	...
Xanthotoxin	1-32	1-35	...
Imperatorin (=Marmelosin)	1-32	1-32	...
Seselin	1-32	1-35	...

It will be seen that none of these compounds possess any hæmostatic activity. The results so far obtained indicate that hæmostatic property is not inherent in the coumarin nucleus, but manifests itself, in a striking manner, only on introduction of an alkoxy group in a suitable position. No effects are observed when the oxygen atom in position 7 forms part of a furan or chromene ring.

It occurred to us that the hæmostatic action might be shared by compounds other than coumarins, but which are genetically related to 7-methoxycoumarin, or which have some close analogy in configuration with it. The following table summarises the results obtained with such compounds.

TABLE VI.

Name of compound.	Normal clotting time (n): Min.-Secs.	Observed clotting time (o): Min.-Secs.	H. A.
2-Hydroxy-4-methoxy-benzaldehyde	1-40	1-22	18
2-Hydroxy-4-methoxy-acetophenone	1-40	1-9	31
2-Hydrovy-3-methoxy-benzaldehyde	2-00	2-5	...
3-Hydroxy-4-methoxy-benzaldehyde	2-00	1-53	...
4-Hydroxy-3-methoxy-banzaldehyde	2-15	2-18	...
4-Methoxycinnamic acid	1-25	1-28	...
4-Methoxycoumaric acid	1-25	1-5	23
4-Methoxybenzoylpropionic acid	2-00	1-54	...
Resorcinol monomethyl ether	Decomposition of RBC		
3-Methoxyphenyl acetate	2-15	1-00	55
3-Methoxyphenyl benzoate	1-57	1-54	...
3-Methoxyphenyl cinnamate	2-00	1-25	29

The above table presents a very interesting picture. Of the four isomeric hydroxymethoxybenzaldehydes, only the 2-hydroxy-4-methoxy compound has got a marked activity. The replacement of the $-CHO$ group of this compound by $-CO.CH_3$ enhances the activity. 4-Methoxycoumaric acid has a definite action. Resorcinol monomethyl ether behaves like phenol in bringing about a deep seated change in blood corpuscles. Its benzoate is inactive, but the cinnamate and acetate are active.

EXPERIMENTAL

The clotting time of blood was determined by means of the technique described in detail in Part I.

Many of the compounds which have been studied in this paper are known. They were either prepared by known methods,or procured from the market. Only new compounds are described below.

Preparation of Alkoxycoumarins.—A general method of preparation consists in the condensation of umbelliferone with the appropriate alkyl halide in molecular proportions in dry acetone in presence of two molecules of anhydrous potassium carbonate. The duration of heating under reflux varied from 4 to 6 hours. The filtered acetone solution was then evaporated to dryness, and the residue was washed with cold water to remove potassium salts. The unchanged umbelliferone was removed by treatment with 1% aqueous alkali. The purified product was then distilled in high vacuum and crystallised from suitable solvents.

7-Ethylumbelliferone (from umbelliferone and ethyl iodide) formed colourless needles, m.p. 89° (dilute alcohol). Yield 85% ; m.p. in literature 88°. Blue violet fluorescence in concentrated sulphuric acid.

7-n-*Propylumbelliferone* (from *n*-propyl iodide and umbelliferone) formed colourless plates from dilute alcohol, m.p. 64°. Yield 80%. Blue fluorescence in sulphuric acid. (Found: C, 70.69 ; H, 5.72. $C_{12}H_{12}O_3$ requires C, 70.60 ; H, 5.88%).

7-iso-*Propylumbelliferone* formed colourless needles, m.p. 57-58°. Yield 82%. Blue violet fluorescence in sulphuric acid. (Found: C, 70.50 ; H, 5.74. $C_{12}H_{12}O_3$ requires C, 70.60 ; H, 5.88%.)

7-n-*Butylumbelliferone*. Colourless prisms from dilute methyl alcohol, m.p. 40°. Yield 75%. Blue fluorescence in sulphuric acid. (Found: C, 71.41 ; H, 6.52. $C_{13}H_{14}O_3$ requires C, 71.55 ; H, 6.42%.)

7-iso-*Butylumbelliferone* formed colourless prisms from dilute methyl alcohol, m.p. 80°. Yield 60%. Blue fluorescence in concentrated sulphuric acid. (Found: C, 71.93 ; H, 6.54. $C_{13}H_{14}O_3$ requires C, 71.55 ; H, 6.42%.)

7-Allylumbelliferone formed needles from ether-petroleum ether, m.p. 86-87°. Yield 90%. Sky blue fluorescence in sulphuric acid. (Found: C, 71.64 ; H, 5.06. $C_{12}H_{10}O_3$ requires C, 71.29 ; H, 4.95%.)

7-Benzylumbelliferone (from benzyl chloride and umbelliferone) formed colourless rectangular crystals from dilute alcohol, m.p. 155°. Yield 60%. (Found: C, 76.12 ; H, 4.48. $C_{16}H_{12}O_3$ requires C, 76.13 ; H, 4.76%.) Violet fluorescence in sulphuric acid.

Bis-umbelliferone-7-ethylene ether (I) from 1 mol. ethylene bromide and 2 mols. umbelliferone. Needles from pyridine-alcohol, m.p. 226°. Yield 50%. Violet fluorescence in sulphuric acid. (Found: C, 68.29 ; H, 3.95. $C_{20}H_{14}O_6$ requires C, 68.57 ; H, 4.00%.)

Bis-umbelliferone-7-trimethylene ether (II) from trimethylene bromide (1 mol.) and umbelliferone (2 mols.). Colourless crystals from pyridine, m.p. 181-2°. Yield 50%. Violet fluorescence in sulphuric acid. (Found: C, 69.47 ; H, 4.44. $C_{21}H_{16}O_6$ requires C, 69.23 ; H, 4.40%.)

Umbelliferone-7-acetic acid was prepared by using molecular proportions of monochloracetic acid and umbelliferone, but an excess of potassium carbonate. The residue after removal of the solvent was acidified with hydrochloric acid. The precipitate was collected and dissolved in dilute sodium carbonate and filtered. The filtrate on acidification gave a precipitate which was crystallised from water. Colourless needles, m.p. 210-12°. Yield 40%. Violet fluorescence in sulphuric acid. (Found: C, 60.19 ; H, 3.48. $C_{11}H_8O_5$ requires C, 60.00 ; H, 3.64%.)

4-Alkylumbelliferone alkyl ethers were prepared by the general method already described. Some of these have already been described by Pfau (2). The *n*-propyl ether melted at 78° (lit. 72-3°) and the *iso*butyl compound had m.p. 57° (lit. 55-6°.)

*4-Methyl-7-*iso*propylumbelliferone* formed colourless needles from dilute alcohol, m.p. 88°. Yield 60%. Violet fluorescence in sulphuric acid. (Found: C, 71.61 ; H, 6.23. $C_{13}H_{14}O_3$ requires C, 71.55 ; H, 6.4%.)

*4-Methyl-7-*n*-butylumbelliferone* had m.p. 51°. It formed thick colourless crystals and gave a violet fluorescence in sulphuric acid. Yield 40%. (Found: C, 72.02 ; H, 6.71. $C_{14}H_{16}O_3$ requires C, 72.41 ; H, 6.90%.)

Daphnetin dimethyl ether was prepared from 1 mol. daphnetin and 2 mols. methyl iodide. Needles from dilute alcohol m.p. 118-9°. Yield 50%. Yellow colour in sulphuric acid. (Found: C, 64.19 ; H, 4.71. $C_{11}H_{10}O_4$ requires C, 64.07 ; H, 4.85%.)

3-Methoxyphenylbenzoate was prepared from resorcinol monomethyl ether and benzoyl chloride. Colourless flakes from dilute alcohol, m.p. 110-11°. (Found: C, 73.67 ; H, 5.10. $C_{14}H_{12}O_3$ requires C, 73.69 ; H, 5.26%.)

3-Methoxyphenylcinnamate was likewise prepared from cinnamoyl chloride and resorcinol monomethyl ether. Colourless plates, m.p. 120-21°. Yield 60%. (Found: C, 75.32 ; H, 5.80. $C_{16}H_{14}O_3$ requires C, 75.60 ; H, 5.5%.)

Resacetophenone monomethyl ether and resorcylaldehyde monomethyl ether could also be prepared by the general method using molecular quantities of methyl iodide and the appropriate phenol.

SUMMARY

The hæmostatic activities of a number of compounds allied to ayapanin (=7-methoxycoumarin or herniarin) have been determined. Of these 4-methyl-7-methoxycoumarin, daphnetin dimethyl ether and 3-methoxyphenyl acetate have been found to possess considerable activity.

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A METHOD FOR THE REMOVAL OF CHLOROPHYLL FROM EXTRACTS OF PLANT MATERIALS

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Investigators of drugs and plant constituents are often confronted with the problem of removal of chlorophyll from extracts of plant materials. During the various treatments to which the extracts must necessarily be subjected, chlorophyll may suffer partial decomposition. During the process of concentration of the extracts, chlorophyll or its decomposition products often form a gummy mass with the resinous or oily constituents present in the extract. This dark green insoluble gummy product often carries down appreciable amounts of those constituents which are being attempted to be isolated in a pure condition. The presence of much chlorophyll in the extract therefore not only interferes with the facile isolation of plant constituents but may also decrease their yields. So far as the present authors are aware no attempts have been made to evolve a suitable method for the removal of chlorophyll from extracts or percolates without materially affecting the yields of the desired products. In the following lines an account of the attempts made to solve the problem is presented.

Of all the solvents, alcohol is most frequently used for the isolation of organic plant constituents, although it has a greater solvent action on chlorophyll. Chromatographic separation of chlorophyll from alcoholic extracts, which must necessarily contain varying amounts of water derived from the plant materials, is not feasible. Charcoal was not found to be effective. The idea of generating a suitable adsorbent in *situ* then occurred to us, and lead sulphide was selected for this purpose. The results of various experiments carried out with lead sulphide are described below.

EXPERIMENTAL

Fresh leaves (2.5 g.) of different plants were extracted successfully with small quantities of boiling commercial methyl alcohol, till they were completely exhausted. The combined extracts were then made up to 50 cc. in each case.

Method I.—To 20 cc. of this extract, 0.5 cc. of lead acetate solution (40 g. lead acetate in 100 cc. water) was added and mixed well. Hydrogen sulphide was then passed through the mixture till the precipitation was complete. The precipitated lead sulphide was then filtered off under suction, and the visual colour of the filtrate as also its flourescence in ultra-violet light was noted.

Method II.—Hydrogen sulphide was passed through 20 cc. of the extract till saturated, and then 0.5 cc. of the lead acetate solution was added with stirring. After removal of lead sulphide the colour of the filtrate as also its fluorescence in ultra-violet light was noted.

The fluorescence (ultra-violet) of a diluted (1:100) solution of the corresponding alcoholic extract was also observed. A comparison of the intensity of fluorescence observed with the filtrates and the diluted solution enabled us to form a semi-quantitative idea of the amount of chlorophyll left after treatments by Methods I and II. The results are given in Table I.

TABLE I.

Twenty cc. of extract represent chlorophyll in 1 g. of fresh leaves. The diluted extract was made up of 0.2 cc. extract and 19.8 cc. alcohol.

Name of plants.	Colour of filtrate		Ultra-violet flourescence of		Dilutéd solution
	Method I	Method II	Filtrate (Method I)	Filtrate (Method II)	(1:100)
Canna sativa	Bright yellow	Bright yellow	*Nil.*	*Nil.*	Distinctly bluish green
Clitoria ternatea	,,	,,	,,	Very weak	,,
Ficus religiosa	Pale yellow	Pale yellow	,,	*Nil.*	,,
Helianthus annus	Colourless	Greenish yellow	,,	,,	,,
Hibiscus rosa-chinensis	Pale yellow	Bright yellow	,,	,,	,,
Impatiens balsam	Colourless	Yellow	,,	,,	,,
Jasminum sambac	Pale yellow	Pale yellow	,,	,,	,,
Nyctanthes arbortristis	Bright yellow	Bright yellow	,,	,,	,,
Tabernæmontana coronaria	Pale yellow	Pale yellow	,,	,,	,,
Tagetes erecta	,,	,,	,,	,,	,,

From the above table it would appear that both the methods are almost equally suitable for removing chlorophyll, and that the amount of chlorophyll left after the lead sulphide treatments never exceeded 0.5% of the amount originally present. In other words the method is efficient to the extent of 99.5%.

Similar experiments were carried out with sun-dried and air-dried leaves. The results are tabulated below. In this case 20 cc. extract represent chlorophyll in 1 g. of the dried material.

TABLE II.

Name of plant.	Colour of filtrate Method I (A)	Method II (B)	Ultra-violet flourescence of (A)	(B)
Alocasia indica	Bright yellow.	Bright yellow	*Nil.*	*Nil.*
Canna sativa	Yellow	,,	,,	,,
Ficus religiosa	Pale yellow	Pale yellow	,,	,,
Helianthus annus	Almost colourless	Almost colourless	,,	,,
Michælia Champaca	Pale yellow	Pale yellow	,,	,,
Raphnus sativus	,,	Yellow	,,	Very weak
Tabernæmontana coronaria	Colourless	Colourless	,,	*Nil.*

The methods are, therefore, applicable to dry materials also.

Having thus demonstrated the suitability of the method, the minimum amount of lead acetate necessary for the complete removal of chlorophyll was determined. Varying amounts of lead acetate solution were added to 20 cc. of extract (= 1 g. dried material). Method I was used and the colour of the filtrate noted.

TABLE III.
Canna sativa extract: 1 cc. lead acetate = 0.35 g.

Lead acetate added.	Colour of filtrate.
0.1 cc.	Distinct green
0.15 cc.	Green, but less intense
0.2 cc.	Faint green
0.25 cc.	Bright yellow
0.3 cc.	,,
0.4 cc.	,,
0.5 cc.	,,

These experiments show that lead acetate equal to 9% of the weight of plant material taken is sufficient for the removal of chlorophyll. Obviously the amount would differ with different plant materials, but not to a large extent, if leaves are used. The minimum amount of lead acetate required can be determined in individual cases, by means a preliminary experiment, and it is advisable not to use a large excess of the acetate.

2

Experiments were then conducted with a view to observe the amount of recovery of an alkaloid and a flavone after the lead sulphide treatment. *Canna sativa* extract (20 cc.) with varying amounts of gardenin and strychnine acetate were subjected to Method I, using the minimum of lead acetate, that is 9%. Results are given in Table IV.

TABLE IV.

Gardenin Added.	Gardenin Recovered.	Strychnine Added (acetate)	Strychnine Recovered (base)
0.15 g.	0.132 g. (88%)		
	0.128 g. (86%)	0.05 g.	0.012 g. (96.5%)
			0.011
0.1 g.	0.08 g. (80%)	0.15 g.	0.097 g. (78%)
	0.092 g. (92%)		0.099
			0.101
0.05 g.	0.042 g. (81%)		
	0.046 g. (92%)	0.1 g.	0.078 g. (95%)
			0.084
0.025 g.	0.018 g. (72%)		

From the data it would appear that the recovery is fairly satisfactory, but in the case of strychnine, the recovery tends to be less than 95% if the concentration of the alkaloid is higher than 0.5% in the extract.

Copper sulphate has also been used in place of lead acetate. But freshly precipitated copper sulphide is not as effective as lead sulphide, inasmuch as 24% of copper sulphate are necessary as against 9% of lead acetate.

SUMMARY

A method for the removal of chlorophyll from alcoholic extracts of plant materials has been evolved. It is simple and inexpensive. It does not seriously interfere with the isolation of active principles of plants.

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INVESTIGATIONS ON THE NEW ANTIDIABETIC PRINCIPLE (AMELLIN). PART VI. ITS ROLE IN THE PREVENTION OF EXCESSIVE PROTEIN CATABOLISM IN DIABETES

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Diabetes mellitus has been classed as one of the wasting diseases, characterised by high urinary nitrogen excretion or by the high level of blood-non-protein-nitrogen. Some hold, however, that there is no appreciable rise in protein catabolism in cases of diabetes (1). But it has been overlooked by them that diuresis helps the system to excrete the large quantity of nitrogenous products formed and maintains the limit of blood non-protein-nitrogen, which is taken as normal by them.

Lauter and Jenke (2) observe that in severe ketosis excretion of nitrogen in urine becomes very great and it has been shown by Bulger *et al* (3) that in most severe stage of acidosis blood protein nitrogen is also elevated thus showing that both the food and body proteins are sacrificed wastefully. It has also been known from the work of Gaebler and Murlin (4) that injection of phlorhidzin, which acts as a kidney poison, causes increase of protein metabolism and excretion of nitrogenous substances in urine. Wilder *et al* (5) have shown that when a protein diet is prescribed for a diabetic individual, his condition soon becomes serious and this can be remedied by substituting the excess protein by carbohydrate and fat. This finding as well as the claims of Petren (6) that a diabetic requires less protein than a normal person, indicate clearly that the metabolism of protein also gets disturbed in case of diabetes mellitus. Wright (7) points out that if a protein meal is given to a starving diabetic D and N excretions are increased but D:N ratio still remains constant. This suggests that both sugar and nitrogen come from the same source *i.e.* the tissue protein.

This present paper will give further confirmation to these findings regarding the condition of protein metabolism in diabetes specially through investigation of the concentration of urea, uric acid and creatinine in urine and urea and creatinine in the blood of diabetics. This will also be shown how the new anti-diabetic principle, amellin, which has been found to reduce hyperglycemia (8) and lipemia (9), can also set excessive protein metabolism in proper order by reducing the concentration of these nitrogenous substances.

Urea, Uric Acid and Creatinine in Blood and Urine

Increase of endogenous urea has long been known to be an indication of destruction of body tissue. Moreover in diseases effecting chiefly the liver or in case of intoxications that bring about destruction of body protein the excessive excretion of nitrogen in urine takes place mainly in the form of urea.

Lennox (10) observes that marked retention of uric acid occurs in nephritis, in gout and during starvation. This signifies its relationship with the endogenous protein catabolism. According to Lennox the retention of uric acid is in some way associated with ketosis which is one of the grave symptoms in diabetes mellitus.

Creatinine output in a normal individual has been found by Folin (11) to be independent of protein intake and maintains a constant level thus serving as an index to tissue catabolism alone. Increase in the concentration of creatinine in progressive loss of renal function has been shown by Myers and his associates (12). That the increased creatinine excretion again results from an increase in blood uric acid has been shown from the work of Abderhalden and Baudze (13). Feeding of Sodiumbicarbonate or disodium phosphate, which can bring about partial neutralisation of the acid products, has recently been found by Beard and his associates (14) to increase the transformation of creatine to creatinine and hence to reduce its concentration in urine and blood.

Amellin has previously been reported to bring about relief in ketosis and acidosis accompanied with diabetes mellitus. Its influence in preventing the tissue catabolism as indicated by lowering the excess concentration of urea and creatinine in blood and of urea, uric acid and creatinine in urine will be shown in the Table I. Regarding excretion of uric acid, however, in case of our patients, its concentration in urine before commencement of amellin treatment was in almost all cases, somewhat below the normal value, which rose up along with the gradual progress of the patients and finally reached the normal limit.

Experimental

Urine Analysis :—

(*a*) Estimation of urine sugar was made according to Benedict's method for its simplicity and accuracy.

(*b*) Urine urea was estimated by the Hypobromite method (15).

(*c*) Urine creatinine was determined by Folin's colorimetric method (16).

(*d*) Estimation of uric acid in urine was made according to the permanganate method (17).

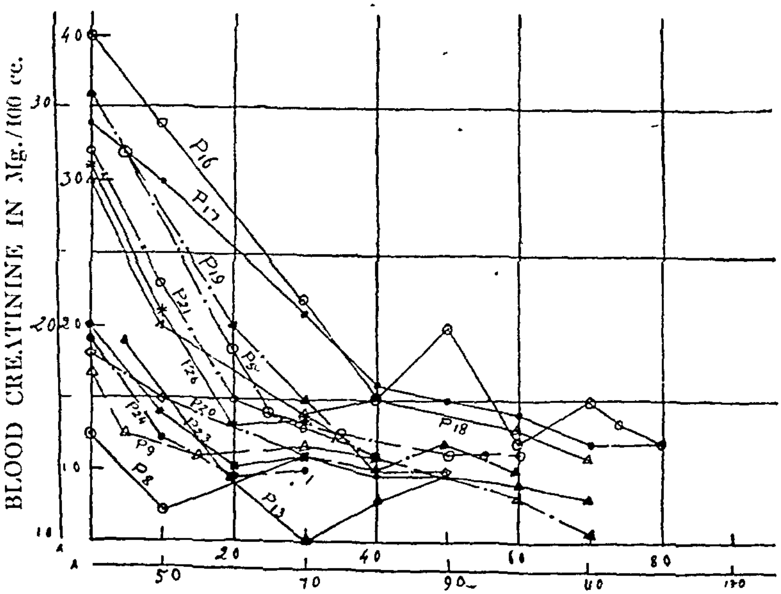
Blood Analysis :—

(*a*) Blood was determined by urease—Nesslerisation method (18).

(*b*) Blood creatinine was estimated by Folin's colorimetric method.

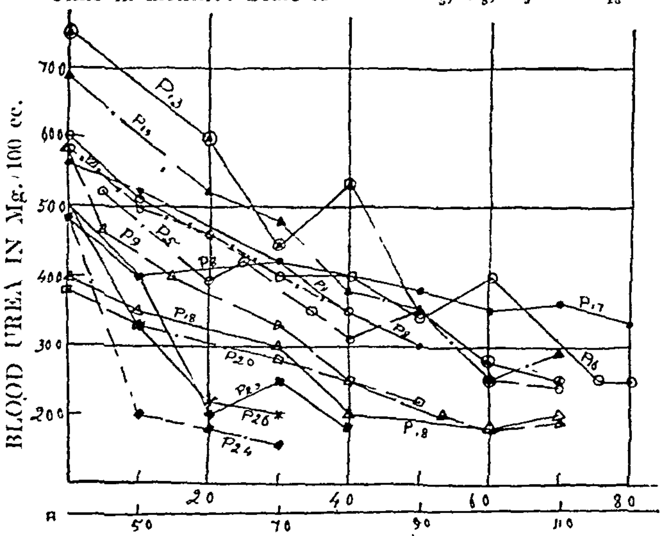
(*c*) For the determination of blood sugar the method of Hagedorn and Jensen (19) was adopted.

All these values as changed through use of amellin are shown in Table I and those of blood creatinine and blood urea are represented graphically in figures 1 and 2 respectively.



Time in months: Scale A → for P_5, P_8, P_9 and P_{13}

FIG. 1



Time in months: Scale A → for P_5, P_8, P_9 and P_{13}

FIG. 2

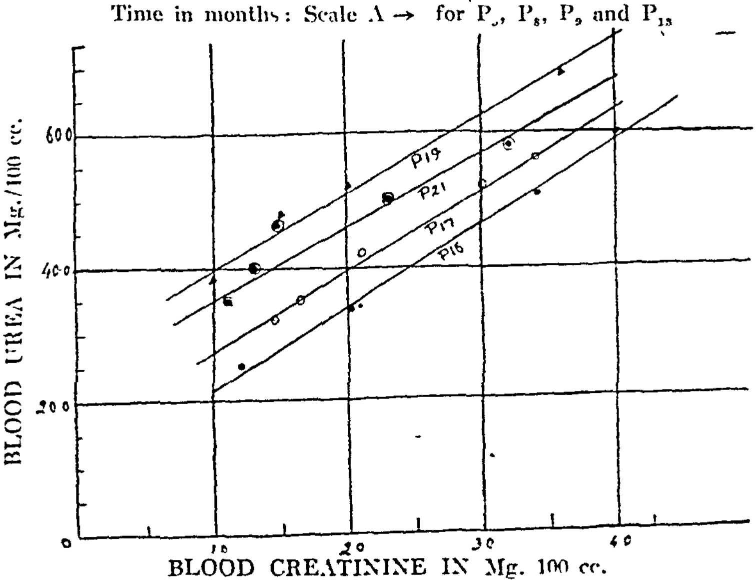


FIG. 3

The results of blood urea have been plotted against those of blood creatinine in figure 3. The curves so obtained indicate clearly some relationship between these two constituents in diabetics while progressing through the effect of amellin, particularly upto certain stage when the virulence of the disease is almost over.

Results obtained from analyses of urine and blood of normal Bengalee youths are represented in Table II.

TABLE I.

Showing the changes in the values of non-protein nitrogenous substances of blood and urine of the diabetic patients during treatment with amellin.

Case No.	Diabetic patients during treatment.	24 hours' urinary.				Blood.		
		Sugar (g.)	Uric acid (mg.)	Urea (g.)	Creatinine (g.)	Sugar (mg. %)	Urea (mg. %)	Creatinine (mg. %)
16.	Before treatment	..	310.0	42.4	5.8	140.4	60.0	4.0
	1 month after treatment	...	348.0	35.0	4.5	126.8	51.0	3.1
	7.5 months ,,	...	800.0	3.6	1.2	100.0	25.0	1.3
17.	Before treatment	201.6	390.0	55.0	5.6	293.0	55.0	3.1
	8 months after treatment	14.8	720.0	20.0	3.0	218.5	35.0	1.4
	13 ,, ,, ,,	*Nil.*	800.0	5.0	1.2	180.0	...	...
18.	Before treatment	108.0	680.0	12.0	3.0	242.0	40.0	3.0
	4 months after treatment	14.3	810.0	10.0	1.1	125.0	20.0	1.5
	7 ,, ,, ,,	*Nil.*	700.0	2.0	1.1	96.0	20.0	1.1
19.	Before treatment	51.0	740.0	45.0	3.6	256.0	69.0	3.6
	5 months after treatment	trace	890.0	8.0	2.3	182.0	35.0	1.2
	7 ,, ,, ,,	*Nil.*	870.0	6.0	1.3	101.0	29.0	1.0
20.	Before treatment	46.5	600.0	20.0	2.8	189.3	38.0	1.8
	3 months after treatment	*Nil.*	750.0	4.0	2.0	123.0	28.0	1.1
	6.5 ,, ,, ,,	*Nil.*	730.0	3.0	1.1	98.0	21.0	1.1
21.	Before treatment	67.5	150.0	36.0	3.0	223.6	58.0	3.2
	4 months after treatment	32.0	670.0	...	1.2	168.0	35.0	1.1
	8 ,, ,, ,,	*Nil.*	...	5.0	1.1	...	...	...
23.	Before treatment	39.0	710.0	25.0	2.4	180.0	50.0	2.0
	4 months after treatment	trace	780.0	6.0	1.1	110.0	18.0	1.0
24.	Before treatment	36.0	1300.0	25.0	2.6	147.0	49.0	1.9
	3 months after treatment	trace	920.0	4.0	1.05	75.0	16.5	1.0
26.	Before treatment	*Nil.*	620.0	18.0	1.4	90.0	50.0	3.1
	3 months after treatment	*Nil.*	800.0	2.0	1.0	74.0	20.0	1.4
5.	6 ,, ,, ,,	18.0	470.0	15.4	1.5	270.0	39.0	1.9
	10 ,, ,, ,,	trace	810.0	7.0	0.98	139.0	24.0	1.45
8.	4 ,, ,, ,,	33.0	800.0	31.0	3.0	218.0	48.0	1.5
	9 ,, ,, ,,	trace	850.0	11.0	1.0	221.0	30.0	1.3
9.	4 ,, ,, ,,	20.0	795.0	20.0	2.2	173.0	58.0	1.8
	11 ,, ,, ,,	*Nil.*	960.0	8.0	1.0	100.0	19.0	1.1
13.	4 ,, ,, ,,	58.8	665.0	40.0	4.0	218.0	75.0	1.0
	11 ,, ,, ,,	trace	900.0	7.0	1.3	116.0	25.0	1.2

TABLE II.

Results showing the values of non-protein nitrogen of blood and urine of normal Bengalee youths.

Name.	Age yrs.	24 hours' urinary.		Blood.	
		Urea (g.)	Creatinine (g.)	Urea (mg. %)	Creatinine (mg. %)
A.S.	21	5.0	1.2	22.0	1.7
S.C.	22	8.0	1.3	25.0	1.5
C.P.	20	13.0	1.3	36.0	1.2
A.B.	23	5.0	1.25	30.0	1.05
M.C.	25	2.0	1.2	30.0	1.0
N.B.	24	4.0	1.1	20.0	0.8
K.M.	20	0.8	1.2	23.0	0.8
N.C.	24	10.0	0.9	12.0	0.95
S.D.	23	3.0	1.15	10.0	1.05
P.R.	27	5.0	0.90	9.6	1.1
H.N.	26	6.0	1.05	13.0	1.2
M.N.	28	8.0	1.10	18.0	1.5
D.D.	20	4.0	1.0	23.0	1.5
M.M.	29	7.0	1.3	35.0	0.9
J.G.	22	3.0	1.2	30.0	1.0
S.H.	24	15.0	1.0	26.0	0.8
S.B.	23	8.0	1.0	12.0	1.1
S.G.	26	5.0	1.1	16.0	0.5
P.G.	20	4.0	0.9	19.0	0.8
J.R.	25	1.0	1.15	28.0	0.5
Average	...	5.84	1.11	21.1	1.01
Maximum	...	15.0	1.3	36.0	1.7
Minimum	...	0.8	0.9	9.6	0.5

Discussion

Though no definite relationship can be established between the values of blood sugar and those of blood non-protein nitrogen of diabetics in general, it has been observed that hyperglycemia is always associated with abnormally high values of blood urea and blood creatinine. Their elimination along with urine is also great. The value of blood urea as well as blood creatinine have been found to be interdependent on each other ; the nature of their fall during the first few weeks of treatment with amellin has been found to be almost similar. In most of the patients. however, the creatinine values reach the neighbourhood of normal limit within three to four months' time, while it requires three to six months or more for urea to come down to the normal range.

The high values of blood non-protein nitrogen in Bengalee diabetics, who are not generally accustomed to high protein diet is also suggestive of severely disturbed protein catabolism. This finding confirms the statements of Wilder (loc. cit.) and Petren (loc. cit.) that a diabetic requires less protein than a normal individual and warrants the diabetics against taking excessive meat, egg or fish diet until the metabolic disturbances are properly restored.

It can also be recorded that none of our diabetic subjects were advised to cut

down their protein diet, which was originally, in almost all the cases, far below the normal requirements ; and even then their non-protein nitrogen values fell considerably through the ingestion of amellin. Amellin thus has claim:

(*i*) to stopping excessive protein catabolism and other disturbances.

(*ii*) to restoring normal metabolism of protein.

To sum up, amellin can prevent tissue wastage in diabetics and effects utilisation of protein, thus indicating the possibility of formation of tissues and regeneration of the degenerated cells in the system. Some idea regarding tissue formation by healing up of wound has previously been reported (8).

Summary

1. Non-protein nitrogen has been studied in the urine and blood of thirteen Bengalee diabetics during their progress through amellin.

2. Urinary elimination of urea, uric acid and creatinine as well as the high values of blood urea and blood creatinine have been found to come down to the normal range within three to six months' time.

3. A linear relationship between the values of blood urea and blood creatinine has been established.

4. The view that high protein diet cannot be beneficial to the diabetics before correcting their metabolic disturbances, has been substantiated.

5. That amellin has a claim to preventing tissue wastage and bringing about utilisation of food protein is established.

Acknowledgement

We should like to express our sincere thanks to Prof. J. K. Chowdhury for his kind encouragement and facilities offered and to Dr. B. C. Roy of Calcutta for his helpful advice and criticism. Thanks are also due to Dr. K. P. Basu for his kind interest in this work.

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PROTHROMBIN IN NORMAL AND PATHOLOGICAL CONDITIONS

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Dam and Co-workers (1-5) observed that chicks on synthetic diets deficient in vitamin K developed hæmorrhages and that this delay in coagulation of blood was found to be due to the deficiency of prothrombin in the blood. They have also shown that vitamin K plays an important rôle in the formation of prothrombin. This discovery led to the study of a new experimental method of great value in investigating the function of vitamin K. Since then the determination of prothrombin in blood plasma acquired considerable importance.

Numerous studies (6, 7, 8) relating to the prothrombin level in health and disease have shown that the prothrombin level is constant in normal individuals and that it decreases in various diseases, particularly those affecting the liver, namely, jaundice, cirrhosis of liver etc. and this hypoprothrombinæmia could be cured by the administration of vitamin K. There is, however, relatively little information available on the prothrombin level in tuberculosis, malaria, anæmia and other allied diseases. The object of the present investigation was to present a simple method of determining the prothrombin level of plasma and to collect data from the determination of the prothrombin content of a large number of normal and diseased persons suffering from tuberculosis, malaria, anæmia, jaundice and other diseases.

EXPERIMENTAL

Determination of Prothrombin.—The method for the determination of prothrombin in blood plasma is based on the principle that the prothrombin

in presence of thromboplastin (thrombokinase and calcium) is converted into thrombin which then reacts with the fibrinogen of the plasma to produce the fibrin clot. The time taken for the formation of the clot after the addition of thromboplastin and calcium to blood plasma is a measure of the prothrombin content of blood. The higher the prothrombin content of plasma the lower is the time taken for the formation of clot.

Extracts from brain, liver and lung are used as the source of thromboplastin (8-13). This technique for the determination of prothrombin by using brain and other tissue extracts as source of thromboplastin appears to possess certain disadvantages. In the first place, the time and the difficulty involved in preparing the tissue extract restrict the use of the method in the routine determination of the prothrombin level. In the second place, the lack of stability of the thromboplastin in tissue extracts (14) necessitates the preparation of fresh extract from time to time and carrying out control tests. In the third place, extracts of constant potency cannot be obtained every time and hence normal figures for prothrombin time should be determined for each extract. The normal value usually, therefore, varies with the extract used, thereby the comparison of the results obtained in different laboratories is rendered difficult and lastly, the recognition of the end point—the formation of a fibrin clot—is rendered difficult due to the milky appearance of the brain extract.

Recently Fullerton (15) has suggested a simple method of estimating prothrombin in which a commercial preparation of Russells' Viper venom (*Stypven*) as the source of thromboplastin was used instead of tissue extract. By using this venom as source of thromboplastin the disadvantages usually encountered in the use of brain extracts are eliminated. In the present investigation the method of Fullerton was adopted with slight modification in the preparation of the thromboplastin solution as suggested by Iyengar *et al* (16).

Reagents required:—

1. Potassium oxalate 0.1 *M*.
2. Thromboplastin-Calcium chloride mixture: (1 in 10,000 solution of Russells' Viper venom in 0.025 *M* calcium chloride solution).

A mixture of Russells' Viper venom (from the Haffkine Institute, Bombay) and calcium chloride was prepared by dissolving the venom in calcium chloride solution.

This mixture is found to be more convenient as suggested by Iyengar *et al* (16) than the use of these reagents separately (15). According to Iyengar the addition of 0.2 cc. of a 1 in 20,000 solution of Russells' Viper venom in 0.025 *M* calcium chloride solution gave a satisfactory result. It reduced the dilution of the prothrombin in the plasma and accelerated the speed of the thromboplastin-prothrombin-calcium chloride reaction. As the solution is quite stable at 5° under toluene, standardised stock solutions could be prepared for routine prothrombin determinations.

In Table I are given figures illustrating the stability of thromboplastin reagent on storage.

TABLE I.

Stability of thromboplastin in the Russells'-Viper venom-Calcium chloride mixture.

Temperature = 5° (in ice room)

Time of storage in days.	Prothrombin time in seconds with the stored venom.	Prothrombin time in seconds with the the fresh venom.	Loss in potency.
0	8	8	0
30	9-10	8-10	0
90	10	8.10	0
140	10	10	0
180	10	10	0

The results show that the thromboplastin reagent is very stable after storage for nearly six months at 5°. It was also found that light has no influence on the potency of thromboplastin in the venom, as the potency of the venom exposed to diffused day-light for 30 days at 5° was not altered.

Procedure.—5 cc. of freshly drawn venous blood were added to 0.5 cc. of potassium oxalate solution. Clear plasma was obtained by centrifugation. 0.2 cc. of whole plasma was taken in a test tube (5 mm. × 5 cm.) and kept at 37° for few minutes. 0.2 cc. of thromboplastin-calcium chloride mixture which was also kept at the same temperature for some time before use, was added into the test tube and the time which elapsed before the fibrin-web formed was noted with a stop watch. The estimations were always carried out in duplicate. The prothrombin time of normal samples was determined side by side for purposes of comparison and standard.

Effect of storage of blood plasma on the prothrombin content.—The effect of storing blood plasma at room temperature on the prothrombin content was studied by keeping plasma at 5° and determining the activity of the prothrombin at known intervals of time. In Table. II are presented the results.

TABLE II.

Prothrombin content of blood plasma on storage.

	Time of storage in hours.	Prothrombin time in seconds.
Experiment I	0	10
	4	11.5
	24	18
	48	25
	62	33
	110	57
Experiment II	0	10
	1	11

The above results indicate that if blood plasma is kept for more than an hour the clotting time increases greatly. It is, therefore, necessary that the test should be performed as soon as possible, after the preparation of plasma

In Table III are presented the results obtained on the prothrombin time of a large number of normal and pathological cases.

TABLE III.

Prothrombin time in normal and pathological conditions.

Nature of disease.	Sr. No.	Name.		Age.	Prothrombin time.	Mean prothrombin time.
Normal cases	1.	A.T.	(f)	25	11 secs.	
	2.	K.V.G.	(m)	35	8	
	3.	M.P.	(m)	25	13	
	4.	S.S.R.	(m)		11	
	5.	C.R.K.	(m)	21	10	
	6.	F.R.	(f)	23	10	
	7.	A.R.	(m)	21	10	
	8.	M.H.	(m)	21	10	
	9.	C.	(f)	40	11	
	10.	M.F.	(m)	20	10	
	11.	T.M.	(m)	22	10	
	12.	Bt.	(m)	29	13	
	13.	S.C.	(f)	57	10	
	14.	Mc.	(f)	31	10	
	15.	N.	(m)		10	
	16.	B.S.	(m)		10	
	17.	J.	(m)	22	12	10 secs.
Tuberculosis	1.	J.M.	(f)	30	13	
	2.	Si.	(f)	20	15	
	3.	S.A.	(m)	40	11	
	4.	D.R.	(m)	26	15	
	5.	S.B.	(f)	16	18	
	6.	T.V.	(m)	24	14	
	7.	S.P.	(m)	26	22	
	8.	An.	(m)	35	20	
	9.	B.B.	(f)	35	20	
	10.	V.P.	(f)	18	17	
	11.	A.R.	(f)	32	20	
	12.	An.	(m)	30	22	
	13.	B.	(f)	28	15	
	14.	R.M.	(f)	22	14	
	15.	K.B.	(f)	19	16	
	16.	A.V.	(m)	22	15	
	17.	S.H.	(m)	24	17	
	18.	K.	(m)	35	20	
	19.	R.K.	(m)	28	16	
	20.	V.	(f)	24	20	
	21.	D.K.	(m)	24	25	
	22.	J.S.	(m)	26	26	
	23.	B.B.	(m)	22	55	
	24.	U.P.	(m)	40	45	
	25.	S.S.	(m)	26	40	
	26.	P.B.	(m)	23	21	
	27.	J.S.	(m)	26	32	
	28.	N.M.	(m)	28	25	
	29.	K.S.	(m)	29	20	
	30.	H.S.	(m)	20	18	
	31.	R.S.	(m)	25	16	
	32.	A.K.	(m)	30	18	

TABLE III.—(*Contd.*)

Nature of disease.	Sr. No.	Name.		Age.	Prothrombin time.	Mean prothrombin time.
	33.	D.K.	(m)	24	25 secs.	
	34.	J.	(m)	39	20	
	35.	A.G.	(m)	48	20	
	36.	A.R.	(m)	46	30	
	37.	F.B.	(f)	27	20	
	38.	P.B.	(f)	20	35	
	39.	Is.	(m)	27	27	
	40.	A.	(m)	26	22	
	41.	S.B.	(f)	24	24	
(hip, early)	42.	Ag.	(m)	40	29	22 secs.
Anæmia	1.	V.	(f)	19	17	
	2.	S.	(f)	25	27	
	3.	D.S.	(m)	23	20	
	4.	A.M.	(m)	40	20	
	5.	R.K.	(m)	26	22	
	6.	A.M.	(f)	60	22	
	7.	S.K.	(m)	21	20	
	8.	M.A.	(m)	22	20	
	9.	N.	(m)	21	25	
	10.	C.Z.	(m)	20	12	
	11.	K.B.	(m)	20	23	
	12.	V.	(m)	20	22	
	13.	K.K.	(m)	20	15	
	14.	G.N.	(m)	18	26	
	15.	D.	(m)	23	15	
	16.	K.D.	(m)	20	21	
	17.	S.N.	(m)	29	20	
	18.	A.L.	(m)	18	21	
	19.	S.R.	(m)	20	20	
	20.	M.H.	(m)	28	18	
	21.	C.R.	(m)	36	16	
	22.	B.R.	(m)	40	15	
	23.	J.S.	(m)	22	19	
	24.	M.S.	(m)	23	23	20 secs.
Malaria	11.	A.	(m)	19	20	
(enlargement of spleen)	1.	A.N.	(m)	20	13	
,,	2.	A.W.	(m)	19	25	
,,	3.	M.G.	(m)	19	20	
,,	4.	H.	(m)	21	20	
,,	5.	H.M.	(m)	25	17	
,,	6.	V.N.	(m)	30	17	
,,	7.	M.D.	(m)	22	18	
,,	8.	D.B.	(m)	20	25	
,,	9.	G.J.	(m)	21	20	
,,	10.	L.C.	(m)	21	22	
,,	11.	R.S.	(m)	23	21	
	12.	J.S.	(f)	31	20	21 secs.
(no enlargement of spleen)	13.	P.V.				

TABLE III.—(*Contd.*)

Nature of disease.	Sr. No.	Name.		Age.	Prothrombin time.	Mean prothrombin time.
Jaundice	1.	T.B.	(m)	19	18 secs.	
	2.	S.K.	(m)	28	19	
	3.	G.D.	(m)	27	16	
	4.	B.B.	(m)	26	12	
	5.	J.	(m)	21	16	
	6.	O.	(m)	19	13	
	7.	S.P.	(m)	25	12	
	8.	M.K.	(m)	22	17	
	9.	T.S.	(m)	14	18	
	10.	A.G.	(m)	20	16	
	11.	N.B.	(m)	22	14	
(associated with pregnancy)	12.	N.N	(f)	16	21	
	13.	M.L.	(f)	24	22	
(associated with catarrh)	14.	M.A.	(f)	19	22	
	15.	D.P.	(m)	21	15	
	16.	S.	(m)	23	24	
	17.	W.	(m)	30	15	
	18.	E.	(m)	30	19	
	19.	Y.	(m)	23	13	
	20.	P.	(m)	36	14	
	21.	A.	(m)	40	15	
	22.	A.S.	(m)	24	15	
	23.	S.F.	(m)	26	13	
(with hepatitis)	24.	W.	(m)	34	15	
	25.	H.	(m)	35	17	
	26.	A.	(f)	20	17	
(with *cirrhosis of liver*)	27.	J.	(m)	23	27	17 secs.
Pregnancy	1.	P.	(f)	27	15	
	2.	R.B.		32	20	
	3.	V.M.		25	15	
	4.	D.		16	15	
	5.	C.		25	13	
	6.	C.M.		20	12	
	7.	S.T.		15	13	
(with albuminuria)	8.	K.		25	6	
(with osteomalacia)	9.	N.		37	20	
	10.	G.		19	25	
	11.	M.N.		38	15	
	12.	E.		31	17.5	
	13.	M.H.		19	18	
	14.	M.B.			21	
	15.	R.			11	
	16.	S.			17	
	17.	W.			15	
	18.	L.			16	
	19.	T.			20	
	20.	C.		23	19	16 secs.

TABLE III.—(*Contd.*)

Nature of disease.	Sr. No.	Name.		Age.	Prothrombin time.	Mean prothrombin time.
Other diseases :						
Pneumonia	1.	S.	(f)	15	15 secs.	
,,	2.	K.	(f)	40	18	
,,	3.	S.	(m)	16	20	
Chronic tonsillitis	4.	Ra.	(f)	14	19	
	5.	Sw.	(f)	13	23	
Cancer (stomach)	6.	B.O.	(f)	67	12	
Cancer (uterus)	7.	P.E.	(f)	48	13	
Neurasthænia	8.	B.R.	(f)	40	25	
General debility	9.	B.A.	(f)	45	20	
High blood pressure	10.	D.	(f)	51	15	
Osteomalacia?	11.	S.M.	(f)	26	17	
Dysentery (bacillary)	12.	S.M.	(f)	17	16	
,,	13.	A.L.	(f)	18	13	
Chronic diarrhœa	14.	F.	(f)	18	20	
Prolapse uterus	15.	C.	(f)	25	11	

Discussion

Prothrombin time in normal healthy persons : The values obtained for the prothrombin time of the blood plasma of 17 normal persons examined range between 8 and 13 seconds, the average value being 10 seconds.

Prothrombin time in various pathological conditions :

1. *Tuberculosis :* Among 42 cases of active and chronic pulmonary tuberculosis studied, a significant deficiency in prothrombin content was found in 30 cases, the prothrombin time being above 20 seconds in all these cases. The average value was 22 seconds.

The study of the relation between prothrombin level and other clinical and roentgenologic status of the patient would be of great interest. The administration of vitamin K in such cases particularly those suffering from pulmonary hæmorrhages with liver damage may have beneficial effect on the patient.

2. *Anæmia :* The prothrombin time of anæmic patients was found to vary between 15 and 22 seconds, the average being 20 seconds. It would be of interest to investigate the effect of administration of liver extract and vitamin K on the prothrombin level in cases of anæmia

3. *Malaria :* In the majority of cases studied there is a definite increase in prothrombin time, the value being above 20 seconds in 10 cases out of a total number of 14 cases. The mean prothrombin time was 21.4 seconds.

4. *Jaundice :* There is hypoprothrombinæmia in jaundice cases investigated. This is in agreement with the results of previous workers.

The finding that hypoprothrombinæmia occurs in various types of diseases in addition to jaundice, namely, tuberculosis, anæmia, and malaria, is of importance in regard to the function of prothrombin in these diseases. In all these diseases the effect of administering vitamin K on the prothrombin level and the general condition of the patient should be investigated.

With a view to find out whether there is any relationship between the plasma prothrombin content and serum calcium, determinations were made of the serum calcium content of each of the samples of blood examined. The results obtained show that there is no significant relationship between these two constituents of blood. The following are the values obtained for the calcium content of the blood serum. Normal—7.2-11.8, average value, 8.8; Tuberculosis—5.3-8.7, average value, 7.15; Anæmia—6.0-12.0, average, 8.5; Malaria—7.0-7.86, average, 7.26; Pneumonia—6.56-8.0, average, 7.28; Jaundice—5.36-11.0, average, 8.6; Pregnancy—4.8-10.6, average, 7.76.

Summary

A simple method for the determination of prothrombin in blood plasma is described in which Russells' Viper venom is used as source of thromboplastin.

The thromboplastin potency of the venom solution is found to be very stable at low temperatures. The venom solution can be kept at ice room temperature (5°) for nearly six months without any loss in potency.

The prothrombin time of normal and healthy persons ranges between 8 and 13 seconds with an average of 10 seconds.

The prothrombin time is markedly prolonged in cases of tuberculosis, anæmia, malaria and jaundice. During pregnancy there is a general rise in prothrombin time.

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VARIATIONS IN THE COMPOSITION OF CROPS GROWN IN DIFFERENT AREAS UNDER VARYING CONDITIONS OF SOIL AND CLIMATE

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McCarrison (1) while attempting to determine the nutritive and vitamin values of rices in common use in India suspected that these might possibly vary with soil conditions. At his suggestion Norris tested and found that the nutritive values of *ragi* (elusive coracana) varied with the manurial treatment of the soil (2).

Viswa Nath and Suryanarayana (3) repeated these experiments in details and came to the conclusion that (*i*) the effect of manuring a crop persisted in the seed and was visible in the next crop when the seed was sown in a soil of moderate fertility, (*ii*) a manured crop gave a seed with better cropping value than an unmanured crop, (*iii*) a crop manured with cattle manure gave a seed with a better cropping value than that from a crop manured with mineral manure or not manured at all, (*iv*) a crop manured with mineral manure gave a seed with a better cropping value than that from an unmanured crop, but the superiority of 'mineral manure seed' over 'No manure seed' varied with the nature of the crop, (*v*) 'Cattle manure seed' was more nutritious than 'No manure seed' or 'Mineral manure seed'. Greaves and Carter (4) also showed that wheat, oats and barley were found to decrease in nitrogen and increase in total ash, phosphorus, calcium and magnesium contents as the irrigation water used in their cultivation was increased.

In the face of the above findings some scientists are of opinion that same varieties and types of different crops when grown under varying soil and climatic conditions have probably different nutritive values. Thus to investigate the variations, if any, in the composition of important crops grown in different areas under different conditions of soil and climate the present investigation was taken up.

Analytical

The methods used were the standard methods for estimation of the different components and the samples used were (I) Bengal gram (T.25) grown at Delhi and Karnal under dry atmospheric condition and at Pussa under wet climatic condition. Although the climate and soil conditions were not much different at Delhi and Karnal the cultivation at the latter place had the advantage of irrigation; (II) Bengal gram (T.53 and T.58) grown at Delhi and Pussa respectively; (III) Bengal gram (T.58) obtained from three different plots at Pussa under same soil and climatic conditions (as control) and (IV) T.4, 6 and 14 of Black gram, S.29 of field pea and T.15, 24, 51, 64 and 80, all grown under different climate and soil conditions at Delhi and Pussa respectively.

Discussion

From Table I it will be seen that T.25 of Bengal gram grown at Delhi, Karnal (Punjab) and Pussa (Bihar) respectively, the Delhi sample had a higher protein content than that of Pussa and a slightly higher one than that of Karnal too. The calcium content also was higher in the Delhi and Karnal samples. Phosphorus and iron, however, were more in the Karnal sample than those from Delhi and Pussa. The fat, carbohydrate and mineral contents were almost equal in all.

The following tables show the composition of the different samples.

TABLE I.

Sample.	Bot. Name	Where grown	Moisture %	Protein %	Fat %	Min. matter %	Fibre %	Carbo-hydrate %	Ca %	P %	Fe mg. per 100 g.
Bengal Gram T.25	Cicer aeritinum	Delhi	11.01	18.98	5.18	2.75	5.23	56.93	0.256	0.210	9.4
		Karnal	11.35	18.00	5.08	2.69	5.76	57.18	0.225	0.278	10.1
		Pussa	12.13	17.02	5.25	2.71	5.88	56.71	0.181	0.216	9.7

TABLE II.

Sample.	Bot. Name	Where grown	Moisture %	Protein %	Fat %	Mineral %	Fibre %	Carbo-hydrate %	Ca %	P %	Fe mg. per 100 g.
Bengal Gram T.53	Cicer aeritinum	Delhi	11.15	18.67	4.96	3.28	5.38	56.26	0.283	0.206	9.32
		Pussa	13.01	17.32	5.02	3.16	5.51	56.05	0.215	0.230	9.68
Do. T.58	Cicer aeritinum	Delhi	11.56	18.85	5.20	2.96	5.63	55,8	0.273	0.201	9.21
CONTROL		Pussa (a)	12.92	17.42	5.01	2.88	5.58	56.16	0.202	0.263	9.58
		(b)	12.88	17.50	4.98	2.86	5.60	56.18	0.201	0.269	9.53
		(c)	12.86	17.46	5.06	2.90	5.53	56.19	0.207	0.266	9.19

TABLE III.

Sample.	Bot. Name	Where grown	Moisture %	Protein %	Fat %		Fibre %	Carbo-hydrate %	Ca %	P %	Fe mg. per 100 g.
Black Gram T.4	Phaseolus Mungo	Delhi	10.87	24.65	1.36	3.40	3.68	56.04	0.268	0.341	9.80
		Pussa	11.90	23.00	1.36	3.45	3.65	56.64	0.206	0.373	9.88
T.6	,,	Delhi	10.76	24.82	1.38	3.37	3.48	56.19	0.253	0.336	9.62
		Pussa	12.10	23.22	1.42	3.42	3.52	56.32	0.201	0.369	10.02
T.14	,,	Delhi	11.12	24.54	1.44	3.03	3.55	56.32	0.250	0.328	9.72
		Pussa	12.08	23.02	1.39	3.09	3.57	56.85	0.206	0.367	9.98
Field Pea S.29	Pisum sativum	Delhi	11.14	22.02	1.14	2.28	4.52	58.90	0.089	0.278	4.01
		Pussa	13.16	20.23	1.17	2.19	4.50	58.45	0.053	0.312	4.87
Red Gram T.15	Cajanus indicus	Delhi	13.10	23.08	1.71	3.86	5.94	52.31	0.188	0.252	8.63
		Pussa	14.33	21.88	1.72	3.72	5.99	52.36	0.156	0.252	9.20
T.24	,,	Delhi	12.96	23.66	1.67	3.83	5.83	52.11	0.193	0.243	8.41
		Pussa	13.88	22.10	1.71	3.74	5.76	52.01	0.152	0.288	9.61
T.51	,,	Delhi	13.22	23.64	1.73	3.76	5.68	51.97	0.198	0.263	8.58
		Pussa	13.84	22.12	1.68	3.78	5.62	51.96	0.163	0.278	9.26
T.64	,,	Delhi	13.16	23.58	1.74	3.72	5.88	51.92	0.178	0.264	8.21
		Pussa	13.96	21.98	1.73	3.75	5.83	52.73	0.161	0.273	9.02
T.80	,,	Delhi	12.82	23.73	1.66	3.82	5.91	52.06	0.186	0.250	8.34
		Pussa	13.97	22.01	1.70	3.78	5.93	52.58	0.168	0.292	8.86

Figures of analysis of T.53 and T.58 of Bengal gram (Table II) also reveal a strikingly higher percentage of total protein and calcium in samples grown at Delhi and higher contents of moisture, phosphorus and iron in those grown at Pussa. There was no *appreciable difference in the fat, carbohydrate* and *mineral* contents of any variety of pulse grown at Delhi and Pussa under entirely different climatic and soil conditions. As a control experiment (last part of Table II) three samples of T.58 grown in three different plots at Pussa under the same climatic and soil conditions were also analysed side by side and the analytical figures showed no significant difference whatsoever in the important proximate principles, *e.g.* proteins, carbohydrates and fats and in the important mineral matters as well.

Analytical figures of T.4, 6 and 14 of black gram, S.29 of field pea and T.15, 24, 51, 64 and 80 of red gram grown at Delhi and Pussa respectively, run almost parallel to the variations already noted in the different varieties of Bengal gram grown at the same places respectively, the protein and calcium being higher in the Delhi samples with less moisture, phosphorus and iron contents. There was practically no difference as regards the two main energy forming proximate principles.

Although Delhi and Karnal have practically the same climate, protein was less but phosphorus and iron more in the Karnal sample than in the Delhi sample. As the cultivation at Karnal is under an irrigated condition these are quite in accord with the findings of Greaves and Carter (*loc. cit*) who are of opinion that under a high irrigation system the nitrogen content of crops like wheat, oat and barley is decreased and that of phosphorus is increased in proportion to the irrigation water used in their production. The calcium content however, contrary to their findings did not show any significant variation.

SUMMARY

(1) The protective principles such as proteins and calcium are higher in the Delhi samples. There is no difference as regards main energy forming principles (fats and carbohydrates) in samples grown at Delhi and Pussa. The moisture, phosphorus and iron are more in the Pussa samples. Regarding the total mineral matters the increase of calcium in the first sample was probably well compensated by the lowering of the phosphorus, iron etc. and *vice versa* (in the Pussa samples) so that there was no significant difference in the total mineral content of the two samples grown under different climate and soil conditions.

(2) The Karnal sample was similar to Delhi sample as regards protein and calcium contents but its phosphorus and iron contents were higher than those of Delhi and Pussa.

Conclusion

Climate and soil condition play important roles in altering the nutritive values of different crops.

Our hearty thanks are due to Rao Bahadur B. Viswanath, F.I.C., Director of the Imperial Agricultural Research Institute, New Delhi for kindly supplying us with the various samples from Delhi, Karnal and Pussa and also for his keen interest in the investigation while it was in progress.

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A COMPARISON OF THE ANTISCORBUTIC VALUES OF SOME COMMON PULSES AND CEREALS IN A SPROUTED CONDITION

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Pulses and cereals, as a rule, contain very little quantity of vitamin C but when they are moistened in water and allowed to germinate the sprouts contain a good supply of this vitamin. In order to study comparatively the vitamin C contents of different pulses and cereals commonly used in India the present work was taken up. The seeds were soaked in water for 24 hours after which water was drained out and the wet seeds were spread out on damp earth and covered with a thick cloth which is kept moist by sprinkling water from time to time. After 48 hours the seeds with beautiful sprouts about half an inch long were taken out for estimation of ascorbic acid.

CHEMICAL ESTIMATION

The method used was Birch, Harris and Ray's modification of Tillman's method (1). The following table shows the comparative values of some vitamin C of some of the commonly used pulses and cereals in a sprouted condition.

TABLE I.

Varieties	Seeds.	Ascorbic acid mg. per 100 g.
Pulses	Bengal Gram (*Cicer æritinum*)	42.6
	Green Gram (*Phaseolus radiatus*)	88.2
	Black Gram (*Phaseolus mungo*)	76.4
	Lentil (*Lens esculenta*)	31.1
Cereals	Wheat (*Triticum vulgare*)	15.0
	Paddy (*Oryza sativa*)	18.7
	Barley (*Hordeum vulgare*)	16.2
	Maize (*Zea mays*)	16.8
	Oat (*Avena sativa*)	5.6

BIOLOGICAL

Biological assay was performed with four groups of 12 guineapigs weighing from 250 to 350 grams each, which developed scurvy on a scorbutic diet for about 45 days. Each animal of the four different batches was given 0.35, 0.4 and 0.45 mg. of pure ascorbic acid respectively in addition to the basal scorbutic diet. After another six weeks' experimental feeding the protective dose for each animal was determined as roughly 0.4 mg. daily.

A second series of another twelve guineapigs with experimental scurvy was again divided into four batches. To each animal of Batch I was given 0.4 mg. of ascorbic acid daily (Control), in addition to the basal vitamin C-free diet, to each of Batch II, 2.2 g. of sprouted paddy, to each of Batch III, 1 g. of sprouted Bengal gram and to each of Batch IV, 0.5 g. of sprouted green gram

in addition to the same basal diet for six weeks. The weight of all the animals, which was going down before, became almost steady after a fortnight and there was some increase in the average weight of all the batches as will be seen from the table below.

TABLE II.

Batch	Diet.	Initial weight, g.	% of increase in weight.					
			1st Week	2nd Week	3rd Week	4th Week	5th Week	6th Week
I	0.4 mg. Ascorbic acid	249	−1.6	+0.8	+2.4	+3.2	+4.2	+6.6
II	2.2 g. sprouted paddy	244	+0.8	+2.0	+2.8	+3.2	+4.5	+6.9
III	1 g. sprouted Bengal gram	239	+0.4	+2.5	+3.6	+4.2	+4.8	+7.1
IV	0.5 g. sprouted Green gram	242	−0.4	+1.2	+3.0	+4.9	+5.2	+7.3

After six weeks all the animals were killed and in none of them any subcutaneous, subperiosteal or subperitoneal hæmorrhage was found on post-mortem examination. Moreover the jaw bones too did not show any indication of scurvy, showing thereby that the scorbutic condition has been completely cured.

So 0.4 mg. of pure ascorbic acid = 2.2 g. of sprouted paddy = 1 g. of sprouted Bengal gram = 0.5 g. of green gram approximately, which roughly corroborate the results of estimation of ascorbic acid in different samples by chemical method.

Discussion

The antiscorbutic values of the four commonly used pulses, green gram, Bengal gram, black gram and lentil (in a sprouted condition) are 88.2, 76.4, 42.6 and 31.1 mg. per 100 g. respectively, whereas those of the sprouted paddy maize, barley, wheat and oat are 18.7, 16.8, 16.2, 15 and 5.6 mg. per 100 g. respectively. So it appears that the lowest of the pulse-group (lentil) contains nearly double of that of the highest amongst the cereal group (paddy). This has been amply corroborated by the biological assay. These data preclude the consideration of sprouted cereals being effectively substituted for sprouted pulses as a source of vitamin C even in the famine areas. Moreover the pulses have another point in their favour that sprouting adds to their flavour and as such they can be easily taken raw without any loss of vitamin C content. On the other hand cereals as a rule require cooking for their proper digestion which is upset by their husk and so there is every possibility of the little ascorbic acid content being lost in the process.

Conclusion

Sprouted pulses as a cheap source of vitamin C may be taken by poor people who cannot afford to take fruits as a protection against scurvy. Even from the point of view of economy the sprouted cereals although cheaper cannot lay any claim as effective substitutes for the sprouted pulses so far as vitamin C is concerned.

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THE NATURE OF IRON IN EGG-YOLK

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Considerable work has been done regarding the iron of the egg. Nearly all the iron of hen's egg is located in the yolk and many believe that it exists in the form of organic complex with protein-like properties. Miescher (1) observed that after prolonged peptic digestion of egg-yolk an insoluble substance separated out, which was found by Bunge (2) to contain iron in organic combination and was named "hæmatogen" by him. Hugounenq and Morel (3) regarded "hæmatogen" as the prosthetic group in the vitellin molecule and the precursor

of the hæmoglobin formed by the chick during incubation of the egg. They found that decomposition by acids leads to the formation of a black pigment containing 2.6% iron.

Hill (4), by applying his own dypyridyl test to egg-yolk, concluded that all its iron is present as inorganic ferric iron and no organic complex exists. Hill estimated the intensity of the red colour which is developed on adding $\alpha:\alpha'$-dipyridyl to the substance after treatment with sodium hydrosulphite in presence of acetate buffer (*p*H 5.5).

Hill's conclusion that the whole of the iron in egg-yolk is present in the inorganic form is based on the following evidence: (*i*) Organo-iron compounds of the hæmatin type do not give the colour reaction by Hill's method, consequently the presence of such compounds in egg-yolk is excluded. (*ii*) The iron obtained by ashing of egg-yolk agrees with the values obtained by Hill's method. Moreover, if Hill's method is employed in an alkaline medium, as for instance, in presence of 10% ammonia solution and hydrazine hydrate as a reducing agent, instead of at the usual *p*H 5.5, the colour is developed with the same intensity. It is not likely, according to Hill, that iron would be liberated from organic combination by alkali.

Tompsett (5) arrived at the conclusion that iron forms complexes with phosphoproteins or phospholipins from the fact that when a solution of ferric salt was added to a suspension of lecithin or solution of caseinogen or to milk and when this was precipitated with trichloroacetic acid, no iron could be detected in the filtrate, but if thiolacetic acid, sodium hydrosulphite or sodium pyrophosphate was added prior to the addition of trichloroacetic acid, the iron could be estimated quantitatively in the filtrate. On the other hand, when mixtures of a ferric salt and either egg-white or a solution of edestin were treated with trichloroacetic acid, the iron could be estimated quantitatively in the filtrate. It has been suggested therefore, that ferric but not ferrous iron forms complexes with organic phosphorus compounds and that upon reduction of the ferric iron by sodium hydrosulphite or thiolacetic acid, these complexes are destroyed. The complexes also appear to be unstable in presence of sodium pyrophosphate which is probably due to the property of iron forming non-ionized compounds with pyrophosphate.

Needham (6) accepts the view of Hill as against the conclusions of Bunge and others, who point out the definite existence of an organo-iron compound called "hæmatogen" by Bunge. All the iron of egg-yolk is not indeed contained in hæmatogen and it is not improbable that colloidal ferric hydroxide may also be present. The *p*H of fresh egg-yolk, according to Aggazzotti (7) is about 4.5, while according to Healy and Peter (8) it is 6.2 to 6.6. The presence of iron as ferric hydroxide is quite possible at the latter *p*H but improbable at *p*H 4.5. Needham (6) and Elvehjem (9) have concluded from Hill's results that egg-yolk contains only inorganic iron.

McFarlane (10) found that lecithovitellin prepared from egg-yolk contains a fair percentage of iron which appears to be present in a very stable combination. He, therefore, concluded that part at least of the iron of the egg is present as organic complex. Commenting on Hill's results, McFarlane says that there is no evidence that $\alpha:\alpha'$-dipyridyl reacts only with inorganic ferrous compounds and does not combine with ferrous organic compounds other than reduced hæmatin and therefore Hill's deduction about the inorganic iron is not justified. The ferrous salt of dipyridyl, according to Blaw (11) is $(Fe(C_{10}H_8N_2)_3-X_2)$, where X may represent a univalent inorganic or organic anion.

In our previous publications (12-16), we have found that about 30-40% of the tissue iron (animal tissue) is present in complex combination with nucleoprotein and copper and this iron can be quantitatively estimated by Hill's dipyridyl method. This complex iron has been found to be a normal constituent of all red-blooded animals hitherto investigated.

A comparative study on the egg-yolk iron and tissue iron (13) reveals some striking differences in their chemical behaviour and as non-avian egg does not contain any nucleoprotein, these difference might be due to the difference in chemical combination.

(1) The amount of iron liberated from the same sample of duck's egg-yolk by the action of sodium hydrosulphite, thiolacetic acid or sodium pyrophosphate is the same and it agrees fairly with the quantity of iron obtained after ashing. The trichloroacetic acid control gives negative test for iron. In the case of fish tissue, the iron value obtained by reduction with sodium hydrosulphite, at *p*H 5.5 is less than that obtained by thiolacetic acid, but if the sodium hydrosulphite is added after the treatment of the tissue with 8% acetic acid by Hill's method as modified by us (12), then the value becomes nearly equal to that obtained with thiolacetic acid. Treatment with sodium pyrophosphate gives only 30 to 40% of the iron in this case. Trichloroacetic acid extract of the control gives the test for iron, the amount being very small. Moreover, the amount of iron obtained after ashing is always greater than that obtained by any of the above methods.

(2) Iron added to egg-yolk cannot be recovered by enzymic hydrolysis, whereas in the case of the fish and other animal tissues, added iron can be quantitatively recovered from the hydrolysate.

(3) Enzymic hydrolysis and increase in acidity do not increase the iron value of egg as in the case of fish and other animal tissues.

The present communication seeks to throw light on this still obscure question of the nature of the total non-hæmin-iron in non-avian egg.

Experimental

Experiments of Fulmer and Jukes (17), Jukes and Kay (18) and McFarlane (10) indicate that about 23% of the iron present in hen's egg-yolk is present in combination with vitellin and copper and this iron can be quantitatively estimated by Hill's dipyridyl method. By applying McFarlane's method (10) for the isoaltion of vitellin-iron-copper (V.I.C.) complex with certain modification, we have found that the whole of the iron present in all egg-yolks hitherto studied, is present in combination with the vitellin fraction of the protein. The method adopted is as follows:

The intact yolks were freed from egg-white by washing first in running water and then under a slow stream of 0.9% sodium chloride solution on filter paper. The vitellin membrane was punctured so that only the yolk contents could be transferred to a beaker without the membrane. The yolk of each egg was diluted to 50 cc. with distilled water. 20 cc. portion of the diluted yolk was taken in a crucible, dried and burnt to white ash for the estimation of total iron. The total iron was estimated by the well-known thiocyanate method described elsewhere (19). For the isolation and estimation of V.I.C. complex another 20 cc. portion of the same egg-yolk was treated as follows and the vitellin iron was estimated by Hill's method (as modified by us).

TABLE I.

Total and Vitellin iron content in 20 cc. portion of diluted egg-yolk, expressed in mg.

Serial No.	Date.	Total iron.	Total vitellin iron.	Sources of iron.
1.	14.3.44	0.570	0.569	Duck's egg-yolk.
2.	20.3.44	0.452	0.441	,,
3.	23.3.44	0.650	0.632	,,
4.	28.3.44	0.580	0.560	,,
5.	29.3.44	0.560	0.532	,,
6.	30.3.44	0.570	0.555	,,
7.	2.4.44	0.490	0.485	,,
8.	10.4.44	0.390	0.391	Hen's egg-yolk.
9.	10.4.44	0.308	0.289	,,
10.	14.4.44	0.300	0.340	,,
11.	15.4.44	0.337	0.340	,,
12.	16.4.44	0.440	0.500	,,
13.	18.4.44	0.290	0.289	Pigeon's egg-yolk.
14.	18.4.44	0.320	0.340	,,

Twenty cc. portion of the diluted egg-yolk was repeatedly extracted with ether until the extracts became colourless. This was carried out in a refrigerator room at -4°C and required 3 to 4 extractions each with an amount of ether equal to the volume of egg-yolk solution. The resultant fat-free yolk was then treated with 60 cc. of 10% sodium chloride solution and well shaken. The mixture was poured into 20 volumes of distilled water, stirred and heated to the coagulation temperature of vitellin (70°C) when the vitellin was completely precipitated. The mixture was allowed to cool, the clear liquid decanted off and the protein precipitate was dialysed in a large parchment dialyser for 24 hours with a few drops of toluene at 10° against repeated changes of distilled water. When the water in the dialyser showed a negative test for chloride, the protein, (in the dialyser) was removed and separated by centrifuging. It was then washed with alcohol and then with ether-alcohol mixture and dried under vacuum. The results are shown in Table I. As the iron content of 20 cc. portion of one egg-yolk of a pigeon was insufficient for the experiment, two eggs were used.

Summary

From the above results, it is clear that the whole of the vitellin iron content of non-avian egg-yolk fairly agrees with the total iron content of the yolk obtained after ashing, indicating that in egg-yolk iron is present in organic combination and not as inorganic iron as stated by Hill (4), Elvehjem (9), Needham (6) and others. This iron can be quantitatively estimated by Hill's dipyridyl method. Experiments on the nature of iron in egg-yolk done by McFarlane gave a low value for V.I.C. iron probably due to the incomplete precipitation of the vitellin protein.

Now the question arises as to whether with the embryonic development of egg, this iron directly goes to form hæmoglobin or *via* iron-copper nucleoprotein complex which has been found to be a normal constituent of all red-blooded animals, birds and fishes hitherto investigated (13 and 14). Work in these lines is in progress. In some of the results in Table I (marked with asterisk) the vitellin iron is slightly higher than the total iron and this is probably due to error in estimation of the total iron.

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INVESTIGATIONS ON MUCUNA SEEDS. PART I. CHEMICAL COMPOSITION AND NUTRITIVE VALUE OF THE SEEDS OF *MUCUNA PRURIENS*, DC.

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Mucuna plants (N.O. *Leguminosae*) may be seen growing in abundance in many parts of India. Prain (1) identified different species of Mucuna viz., *Mucuna monosperma, DC., Mucuna gigantea, DC., Mucuna pruriens, DC., Mucuna utilis, Wall* and *Mucuna nivea, DC.* in Bengal. Of these *Mucuna pruriens* is the most common and is widely used in indigenous medicine. According to Ayurveda, *alkushi* (*Mucuna pruriens*) is highly provocative of sexual vigour, pleasant to the taste, nourishing, bitter, curative of gout and can allay phelgm and plethora. The seeds are said to be highly aphrodisiac.

Very little work has been done on the constituents and properties of this important medicinal plant. Dymock (2) reported some observations on the seeds of *M. pruriens*. The composition of hairs of mucuna pods has been determined by Calvino (3). Lewkowitsh (4) has reported the constants of a mucuna oil from the seeds of *Mucuna capitata,* Dc. occurring in the Dutch Indies. Recently Damodaran and Ramaswamy (5) have isolated 1-3:4-dihydroxyphenylalanine in large amount from the seeds of *M. pruriens, DC.*

The present author has undertaken a systematic investigation of *M. pruriens* seeds including their chemical examination, the determination of nutritive value of the proteins and finally an examination about the aphrodisiac property so widely believed.

Experimental

About 20 kgm of *Mucuna pruriens* seeds, obtained from a local dealer were decorticated and then ground in a flour mill. This powder served as the material for experiments stated here. The seeds were found to contain moisture 9.1%, protein 25.03%, crude fibre 6.75%, petroleum ether extractines 2.96% and ash 3.95%. Qualitative analysis showed that the ash contained a number of mineral elements and plenty of carbonates. Some of the important constituents were estimated and gave the following figures based on the seeds: calcium 0.159%, phosphorus 0.471%, magnesium 0.206%, iron 0.022%, manganese 0.008%, sulphur 0.232% and silica 0.617%.

In order to get an idea regarding the general characters of the constituents present, powdered seeds were successively extracted with different solvents in the usual way and the amounts of extractives obtained in each case were as follows: petroleum ether 2.96%, ether 0.09%, alcohol (rectified spirit) 2.79%, water 32.68%.

The petroleum ether extract contained chiefly an yellow oil mixed with some resinous bodies and organic phosphorus compounds. The ether extract contained chiefly a glucoside which is being investigated in details. In addition, it contained some phospholipids and a small amount of gallic acid. The alcohol extract was deep yellow and on exposure to air turned black. Detailed work with this extract is in progress.

The chemical analysis reveals that seeds contain a large amount of protein and in view of the widely believed nutritive properties of these seeds it was thought desirable to determine the biological value of the proteins present.

The biological value of the seed proteins was found out first by the balance sheet method. The technique employed was that followed by Basu *et al* (6). The metabolic nitrogen, however, was calculated as being directly proportional to food intake. Determinations of biological value were carried out at different levels of protein intake. In Table I the compositions of different diets used in this investigation are given.

TABLE I.

Showing the composition of different diets.

Constituents.	N-free diet.	per cent Protein content of diet.		
		5%	10%	15%
Decorticated mucuna powder	...	200.0 g.	400.0 g.	600.0 g.
Chopped sugar	90.0 g.	90.0	90.0	90.0
Clarified butter	100.0	94.0	88.0	82.0
Codliver oil	20.0	20.0	20.0	20.0
Salt mixture	50.0	50.0	50.0	50.0
Ca-carbonate	8.0	8.0	8.0	8.0
Corn starch	735.0	541.0	347.0	153.0

Data obtained in experiments with nitrogen-free diet are shown in Table II. In Tables III and IV are shown relevant data for metabolism experiments with diets containing 5% and 10% protein. Experiment carried out at 15% level of protein intake did not give any conclusive result on account of very unsatisfactory physical condition of rats. This was probably due to over-ingestion of a toxic principle, l-3:4-dihydroxyphenylalanine, in which the seeds are very rich. For this reason data for this experiment are not reported here.

TABLE II.

Showng experiments with Nitrogen-free ration.
(figures for intake and excretion represent daily averages)

Rat. No.	Body weight	Food intake	Urinary nitrogen	Metabolic nitrogen	Metabolic nitrogen per g. of food
1M	236.0 g.	13.20 g.	41.76 mg.	18.61 mg.	1.41 mg.
2M	235.0	11.00	44.80	16.28	1.48
3F	196.0	12.10	32.60	13.55	1.12
4F	209.0	12.20	32.50	16.56	1.36

TABLE III.

Showing the biological value of Mucuna pruriens seed proteins at 5% protein level.
(figures for intake and excretion represent daily averages)

Rat No.	Body weight in g.	Food intake in g.	Nitrogen intake (mg.)		Urinary nitrogen (mg.)			Fæcal nitrogen (mg.)			Biological value.	Mean B.V.
			Total.	True.	Total.	Endogenous.	True.	Total.	Endogenous.	Exogenous.		
1M	235.0	14.90	119.2	93.99	82.41	41.76	40.65	46.22	21.01	25.21	57	
2M	234.5	14.10	112.8	96.39	81.06	44.80	36.26	37.27	20.86	16.41	62	
3F	196.0	13.80	110.4	96.74	71.32	32.60	38.72	29.11	15.45	13.66	60	61
4F	208.0	12.90	103.2	93.50	65.30	32.50	32.80	27.24	17.51	9.70	65	

TABLE IV.

Showing the biological value of Mucuna pruriens seed proteins at 10% protein level.
(figures for intake and excretion represent daily averages)

Rat No.	Body weight in g.	Food intake in g.	Nitrogen intake (mg.)		Urinary nitrogen (mg.)			Fæcal nitrogen (mg.)			Biological value.	Mean B.V.
			Total.	True.	Total.	Endogenous.	True.	Total.	Endogenous.	Exogenous.		
1M	236.0	14.80	236.8	210.51	125.25	41.76	83.49	47.12	20.86	26.26	60	
2M	235.0	13.20	211.2	191.20	131.65	44.80	86.85	36.51	19.51	17.00	55	
3F	196.0	14.10	225.6	212.35	125.00	32.60	92.40	29.01	15.79	13.25	56.5	55
4F	209.0	12.90	206.4	191.34	128.49	32.50	95.99	32.60	17.54	15.06	50	

It appears from the data submitted that the biological value of the proteins of the seeds is fairly high at low levels of protein intake. At 5% level the value is 61 but at 10% level it is definitely lower, 55. At 15% level the condition of rats was not at all satisfactory as stated before.

In view of the interesting result obtained by the balance sheet method it was thought desirable to see if the seed proteins of *Mucuna pruriens* possessed any growth promoting values. Experiments were carried out with young albino rats of about 30-40 g. weight—the technique followed being that adopted by Basu *et al* (*loc. cit.*).

Experiment at 5% level shows that the rats could maintain the original body-weight for some time but ultimately lost weight slowly. In other words no growth takes place at 5% protein level. These data are, therefore, not recorded here. At 10% level (Table V) the seed proteins exhibited some growth-promoting property and the biological value after 4 weeks was 0.76 but this dropped down to 0.59 after 8 weeks. At 15% level (Table VI) there was a marked drop in the biological value even after only 4 weeks due probably to over-ingestion of l-3:4-dihydroxyphenylalanine. The experiment was, therefore, not continued after 4 weeks.

TABLE V.

Showing the biological value at 10% level of protein intake after 4 weeks.
(figures for intake and excretion represent daily averages)

Rat No.	Initial wt. (g.)	Final wt. (g.)	Total gain in wt. (g.)	Total protein intake (g.)	Biological value.	Mean B.V.
1M	30.0	16.5	16.5	19.4	0.85	
2M	31.0	46.5	15.5	20.2	0.76	
3F	33.0	48.5	15.5	20.8	0.75	0.76
4M	35.0	50.0	15.0	21.5	0.70	
After 8 weeks.						
1M	30.0	55.5	25.5	12.5	0.60	
2M	31.0	56.0	25.0	44.3	0.56	
3F	33.0	59.0	26.0	43.6	0.60	0.59
4M	35.0	60.0	25.0	40.5	0.61	

TABLE VI.

Showing the biological value at 15% level of protein intake after 4 weeks.
(figures for intake and excretion represent daily averages)

Rat No.	Initial wt. (g.)	Final wt. (g.)	Total gain in wt. (g.)	Total protein intake (g.)	Biological value.	Mean B.V.
5F	31.0	42.0	11.0	28.5	0.4	
6M	30.0	47.0	17.0	28.0	0.6	
7M	34.0	52.0	18.0	36.1	0.5	0.47
8M	37.0	51.0	14.0	34.2	0.4	

SUMMARY

Seeds of *Mucuna pruriens,* DC. have been analysed. They contain phosphorus, calcium, iron and manganese..

The petroleum ether extract contains chiefly oil, and in the ether extract a glucoside has been found.

Biological values of the seed proteins have been determined by the balance sheet as well as the growth method. By the balance sheet method the value is 61 at 5% level of protein intake and 55 at 10% level.

Experiments at 5% level by the growth method show that rats cannot maintain their body-weight for a long time. At 10%, however, the biological value after 4 weeks of feeding is 0.76 which drops down to 0.59 after feeding 8 weeks. At 15% level the value is distinctly lower (0.47 only) after 4 weeks.

As the seeds contain a fairly large amount of l-3:4-dihydroxyphenylalanine which appear to be toxic, ingestion of a large quantity of seeds cannot be recommended.

Further work is in progress.

My best thanks are due to Prof. J. K. Chowdhury for his kind interest.

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OBSERVATION ON THE ASSAY OF INSULIN IN INDIA BY THE 'RABBIT METHOD'

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INTRODUCTION

The importance of the accurate standardisation of insulin is recognised by all clinicians. Differences of 10 per cent or so in the potency of a particular brand of insulin may have serious consequences for diabetic patients. Now that attempts are being made to manufacture insulin for the first time in India, the need for its standardization becomes all the more important.

The assay of insulin by the rabbit blood-sugar method was introduced as early as 1926 by Marks (1). Considerable work has since been done on the subject by Hemmingsen and Marks (2), Hemmingsen (3), Hemmingsen and Marks (4), and by the other workers on behalf of the Health Organisation of the League of Nations, (5). The performance of the test has been described in detail by Burn (6). For the convenience of the workers undertaking the assay of Insulin, it is reproduced here in brief.

Rabbits with large ears weighing between 1.5 to 2 kg are chosen for the experiment. Their diet consists of gram, bran, carrot and cabbages. About 12-20 rabbits are taken for each test. They are prepared for test by taking away food from their cages in the afternoon of the day previous to the test. Water is left in the cages. On the next day, they are divided into two equal groups. Each rabbit of the first group receives an injection subcutaneously of one unit of the International standard Insulin and each of the second group receives such a dose of the unknown as may be expected to contain one unit of activity. The volume of the solution injected is about 0.5 cc in both cases. Samples of blood from each rabbit are obtained before the injections of Insulin are made. The fasting blood-sugar contents of these samples are determined. At hourly intervals for five hours after the injection, samples of blood are withdrawn from each rabbit. The

five samples of blood from each rabbit obtained at hourly intervals are pooled by putting 0.1 cc of blood from each hourly sample into a test tube containing 4 c.c. of solution (A), so that at the end of the experiment, each test tube contains 0.5 cc of blood which would represent the average of the five samples. The blood sugar of these pooled samples is determined.

The cross over test. On the first day of test, half of the rabbits are injected with 1 unit of standard insulin and the other half are injected with the unknown sample. On the second day of the test, the groups are crossed over so that those which received the standard now receive the unknown, and those which received the unknown are injected with the standard.

If the initial fasting blood-sugar is X, and the average of the five bleedings after insulin injection is Y, then the percentage of blood-sugar reduction is $\frac{X-Y}{X} \times 100$. The average percentage of blood-sugar reduction for rabbits on standard insulin and the corresponding average for rabbits on a similar dose of the unknown sample, are calculated and compared with each other. If the two figures differ from each other by more than 15 per cent, the dose of the unknown is altered to such an extent as to bring the effects of the standard and sample nearer each other. When the comparative effects of the standard and sample differ from each other by less than 15 per cent, the following formula is applied in order to arrive at the exact potency of the unknown sample.

$$\text{Log. potency of the sample} = \text{Log. assumed potency} + \frac{2}{3}\left\{ \frac{\text{Effect of standard}}{\text{Effect of unknown}} - 1 \right\}$$

The assumed potency is usually the strength stated on the label, which is tacken to be correct for deciding the dose of the unknown sample to be injected. If the effect of the sample (the dose being given on this assumption) differs widely from the effect of the standard, it must be concluded that the potency declared on the label is wrong. From the results of the first experiment the worker can assign an aproximate potency to the sample and on this asumption can alter the dose of the unknown in order to get an effect comparable with that of the standard. When this is done, "the assumed potency in the above formula" will be the strength assigned by the worker. The exact potency is then calculated from the above formula.

Correction for the initial blood-sugar:—Hemmingsen and Marks (*loc. cit*) have shown that the figure for the percentage blood-sugar reduction increases with increasing initial value of the blood-sugar. Hence if the initial blood-sugar values are higher for the unknown sample than for the standard, the percentage reduction figures for the sample will be too high. The converse is also true. The best approach to a correction is to find the difference between the total figures for the initial values, to multiply this difference by 0.22 and to add the result to the total of the percentage reductions on the side showing the lower total value of initial blood-sugar.

Determination of blood-sugar:—The procedure used by Marks (7) for determining blood-sugar is a combination of Somogyi's (8) method of precipitating the proteins with Hagedorn and Jensen's (9 and 10) method of estimating sugar.

Solution A.

Zinc sulphate ($ZnSO_4$, $7H_2O$)	25 g.
N. sulphuric acid	62.5 cc.
Distilled water	to 2,000 cc.

Solution B.

N. sodium hydroxide	100 cc.
Distilled water	to 2,000 cc.

This solution must be adjusted so that 8 cc. exactly neutralizes 1 cc. solution A.

When Solution A is mixed with Solution B in proportion of 1 to 2, the mixture should not be red to phenolphthalein, and the clear supernatant fluid should not give a further precipitate of zinc hydroxide on the addition of more of solution B.

Solution C.

Potassium ferricyanide	0.825 gm.
Anhydrous sodium carbonate	5.3 gm.
Dissolved in distilled water and diluted	to 1,250 cc.

Solution D.

Acetic Acid (glacial)	3 cc.
Distilled water	to 100 cc.

Solution E.

10 cc. of solution E_1 to 90 cc. of solution E_2.

Solution E_1.

Potassium iodide	20 gm.
Distilled water	to 100 cc.

Solution E_2.

Zinc sulphate	25 gm.
Sodium chloride	125 gm.
Distilled water	to 150 cc

Solution F.

N/10 sodium thiosulphate	5 cc.
Sodium bicarbonate	0.01 gm.
Distilled water	to 100 cc.

Starch indicator is prepared by making 1 gm. soluble starch into a paste with a little distilled water and pouring into 100 cc. boiling saturated NaCl.

Procedure:—4 cc. solution A is put in a test-tube and 0.5 cc. blood is delivered into it. 8 cc. of solution B is added, the test-tube is shaken and placed in a boiling water bath for 2-3 minutes. The liquid is filtered through a dry paper. Take 5 cc. filtrate and 5 cc. solution C in a test-tube with a small glass funnel resting on the top to minimize evaporation. Place in a boiling water bath for 15 minutes. Cool, add 2 cc. solution D and 3 cc. solution E. Titrate with solution F from a burette, using the starch as indicator.

The blank titration, when this procedure is followed without the addition of blood filtrate, *i.e.* in the absence of sugar, should be precisely 2 cc.

The following table gives the percentage of sugar in the blood corresponding to amounts of thiosulphate used less than 2 cc.

TABLE I.

Determination of blood-sugar.

cc. $\frac{N}{200}$ thiosulphate	Percentage blood-sugar.
0.0	192.5 mgs.
0.1	177.5
0.2	165.5
0.3	155.0
0.4	145
0.5	135
0.6	125.5
0.7	116
0.8	106.5
0.9	97.5
1.0	88.5
1.1	79.5
1.2	70.5
1.3	62.0
1.4	53.0
1.5	44.0
1.6	35.0
1.7	26.0
1.8	17.0
1.9	8.5

An examination of the methods employed (Quarterly Bulletin, League of Nations, 1936) reveals certain differences between those of European and American workers. In the Insulin Committee Laboratory in Toronto, and in the Lilly Research Laboratories, U.S.A., the dose of insulin ordinarily given is 2 units per rabbit of 2 kg., whereas in the National Institute for Medical Research, London, and other European laboratories, the dose is 1 unit per rabbit or approximately 0.5 unit per kilo. In the American continent, the bleeding of the rabbit is carried out at intervals of $1\frac{1}{2}$, 3 and 5 hours after the subcutaneous injection of Insulin, and a sugar determination on the pooled filtrates from the three samples is made. In Europe however, the bleeding is carried out at hourly intervals and the sugar content of the pooled sample is determined. These two methods have been compared in the present paper.

Marks (11) has determined the relation between dosage and hypoglycemic effect and has found that when doses above 0.75 unit per kilo are given, there is no further increase of effect. Higher doses produce effects which are not proportional to the doses and therefore do not give results of value in determining the potency of the unknown sample. Burn (*loc. cit*) is of opinion that the dose used in Toronto is too high unless rabbits in Toronto are much more resistant to Insulin than rabbits in London. Data relating to dose and response for the rabbits in America are not available and it is therefore not possible to form an opinion as to whether the dose used in Toronto is too high. If there is a possibility of differences in sensitiveness of rabbits, it is desirable to study the response to insulin of rabbits in India in order to fix a suitable level of dosage for use in routine assays in this country.

Hemmingsen and Marks (*loc. cit*) have worked out the relation between average fall of blood sugar during the 5 hours following the administration of insulin and the initial blood sugar level. They have found that the absolute fall tends to increase with increasing initial values. It is therefore of importance to determine the fasting blood sugar of a large number of rabbits in India. This has also been carried out.

Experimental

Rabbits varying in weight from 1.5-2.0 kg., were chosen irrespective of breed, sex, or colour. They were kept under laboratory diet for at least 7 days before experiments were started. The animals were prepared for the blood-sugar determination by keeping them without food for at least 20 hours. Care was taken to eliminate abnormally sensitive and insensitive animals as suggested by Hershey and Lacey (12).

Table II gives the blood-sugar levels of 40 normal rabbits.

TABLE II.

Rabbit No.	Fasting Blood sugar mgs/100 cc.	Rabbit No.	Fasting Blood sugar mgs/100 cc.
1.	108	21.	118
2.	95	22.	125
3.	116	23.	135
4.	89	24.	118
5.	93	25.	116
6.	112	26.	116
7.	105	27.	125
8.	108	28.	130
9.	110	29.	110
10.	138	30.	122
11.	127	31.	115
12.	140	32.	125
13.	145	33.	108
14.	145	34.	112
15.	115	35.	118
16.	120	36.	128
17.	90	37.	135
18.	155	38.	114
19.	150	39.	95
20.	145	40.	82

Average :—118.8 mgs. ± 13.4

The average initial blood-sugar was found to be 100 mgs. by Hemingsen in Copenhagen while Marks working in London found it to be 118 mgs. which corresponds to the average value obtained by us. The normal variation of the fasting blood-sugar is between 80 and 120 mgs. per 100 cc. The values obtained by us show that quite a large proportion of rabbits were at the higher range as will be evident from the frequency distribution (Percentage of rabbits with fasting blood sugar between 80 to 120 = 58 ; percentage of rabbits with blood sugar above 120 = 42). Although the average value is 118.8, a positive tendency towards a higher value than those obtained by workers in Europe, is observed in India. On account of this increased fasting blood sugar, the response to insulin would be expected to be slightly higher than those obtained by other workers. (Hemmingsen and Marks, 4).

Table III gives the relation between dose of an International standard of crystalline insulin and the percentage of blood sugar reduction as estimated on a pooled sample of 5 successive hourly bleeding.

TABLE III.

Dose : unit/kg.	0.25	0.5	0.75	1.00	1.50
No. of Rabbit.	Blood sugar reduction %	Blood sugar reduction %	Blood sugar reduction %	Blood sugar reduction %	Blood sugar reduction %
1.	18.4	38.5	41.5	47.8	47.8
2.	22.5	32.6	46.8	42.5	19.1
3.	25.6	41.5	32.9	56.1	55.6
4.	17.5	46.4	45.8	48.9	56.2
5.	20.4	30.8	31.2	41.9	39.1
6.	15.2	38.2	41.4	51.5	51.5
7.	27.5	28.2	48.9	56.8	46.9
8.	25.5	20.1	36.4	55.2	49.8
9.	20.8	45.8	28.5	18.2	55.8
10.	24.2	41.2	49.8	50.1	58.4
11.	26.5	37.9	51.8	52.5	57.5
12.	14.8	29.8	51.8	46.8	55.2
13.	18.1	38.4	50.2	52.2	51.1
14.	26.4	41.5	42.8	41.4	51.5
15.	17.8	41.8	32.5	16.8	19.5
16.	32.5	28.5	38.2	44.2	47.9
Average reduction %	21.5	36.5	42.3	49.1	51.6
Corresponding figures of Marks	20	33	41	44	45

The difference in the findings is comparatively small and might be attributed to the differences in initial sugar content.

The dose-response curve shows that when doses above 1 unit per kg. are given there is no further significant increase in effect. When the dose of 1 unit per kilo is given the effect is nearly maximal.

A comparison was made as to the figures of blood sugar reduction obtained by the use of the different methods of pooling employed by British and American workers respectively, that is, by (*a*) estimation on a pool of 5 hourly samples

and (b) on a pool of samples taken at $1\frac{1}{2}$, 3 and 5 hours. The results are shown in Table IV, the dose employed being 0.5 unit per kilo.

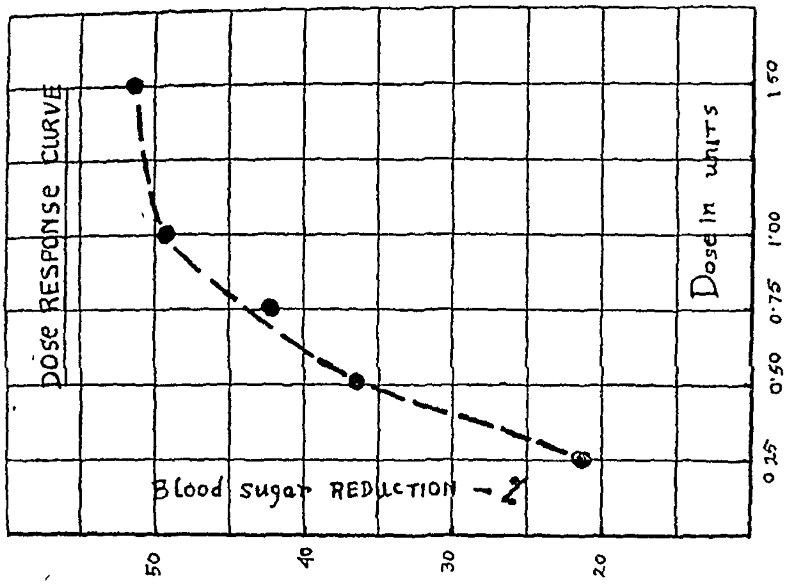

TABLE IV.

Percentage of blood sugar reduction in the hourly pool. (British Method)	Percentage of blood sugar reduction in the pool of $1\frac{1}{2}$, 3 and 5 hours. (Toronto Method)
27.2	27.5
31.5	32.8
25.8	37.8
36.1	21.9
41.5	30.5
46.5	32.8
41.2	21.4
30.5	21.5
30.8	36.5
41.5	26.8
37.9	37.1
29.8	31.7
$m_1 = 35.1 \pm 1.85$	$m_2 = 30.1 \pm 1.72$

Significance Test.

Square of standard Error $E_1^2 = 3.423$

Square of standard Error $E_2^2 = 2.958$

$$T = \frac{m_1 - m_2}{\sqrt{E_1^2 + E_2^2}} = \frac{5.0}{\sqrt{6.381}} = 1.978$$

Probability of error of sampling is about 0.05.
The difference can, therefore, be regarded as significant.

The fall of blood sugar, as determined in the pool obtained by hourly bleeding, represents a better average reduction during the whole period of the experiment than the one determined in the pool obtained by the bleeding at intervals of $1\frac{1}{2}$, 3 and 5 hours after insulin injection ($t=1.978$, $P=0.05$). Nevertheless the smaller number of bleedings at slightly larger intervals would be a great convenience, particularly when 10 or more rabbits have to be experimented upon at the same time. In assaying samples of insulin, the effects are always compared at the same time with those produced by the International standard of insulin on an equal number of rabbits. As the intervals of bleeding will be the same in both groups, the responses measured will be strictly comparable and hence assessment of potency will not be affected. If, however, the difference between the results obtained by the two methods of pooling had been greater, there would have been less justification for adopting the American method. The effect as measured by the American method, is about 30% blood sugar reduction with a dose of 0.5 unit per kg., which is still in the region of proportionality between dose and effect. Hence the dose of 0.5 unit per kg. is considered suitable.

SUMMARY

1. The fasting blood sugar of rabbits in India (irrespective of breed, colour and sex) has been determined in 40 rabbits and the average value is found to be 118.8 mgs. per 100 cc. with a standard deviation of 13.4. The values in 42 per cent of the animals tend to be higher than 120 mgs. per 100 cc.

2. The response of these rabbits to graded doses of insulin has been determined. A dose of 0.5 I.U. per kilo is found in Indian rabbits to give a submaximal hypoglycæmic effect and at this level, a suitable proportionality between dose and response is obtained.

3. The responses to the same dose of Insulin, as measured by the British method of pooling equal quantities of hourly samples of blood and by the Toronto method of pooling from three bleedings at intervals of $1\frac{1}{2}$, 3 and 5 hours have also been determined. The latter method of pooling gives a slightly lower percentage of blood sugar reduction than the former.

4. As the American method of pooling involves a smaller number of bleedings, it is considered suitable for adoption in assaying samples of insulin in India. The accuracy of estimation of potency is not affected since the same intervals of bleeding are adopted for rabbits injected with standard insulin, the two groups of experiments being carried out simultaneously.

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THE STABILITY AND ABSORPTION OF THE ANTIGENS OF BACT. TYPHOSUM WITH SPECIAL REFERENCE TO ORAL IMMUNIZATION. PART I

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(Received for publication, December 27, 1944)

Immunizing value of oral vaccine with Bact. typhosum and the immunity produced by such vaccination in experimental animals as well as in human subjects have long been advocated (1). Prophylactic properties of such vaccination against typhoid infection have been tried in mass experiments and oral vaccines are being utilised by many for the purpose of prophylaxis, as alternative to injectable vaccines. Oral vaccines have also been tried for curative purposes and recently there is a growing tendency to adopt them in general practice.

There still remains great diversity of opinion regarding the precise mode of action of oral vaccines and very little work has been done to follow the process of changes and the fate of the antigens of the oral vaccines in the gastro-intestinal tract as well as their absorption and utilisation in the system in the production of immunity. The evidence so far recorded is indirect as demonstrated by the development of antibodies in the serum or resistance acquired by the vaccinated animal or human subjects to subsequent infection by the same organisms. While Besredka and his group think that the immunity produced by oral vaccine is purely 'local' in the receptive cells of the intestine, the other workers including Topley and Wilson believe that the immunity reaction after oral immunization is 'general' and accompanied by appearance of humoral antibodies. But one point remains clear that whatever may be their mode of action, there can be no doubt that the antigens of Bact. typhosum must reach the intestinal mucosa in an unaltered state before they could stimulate the production of immunity, whether 'local' or 'general' or both.

The rapidly increasing incidence of typhoid fever in this country and special grounds for a wider adoption of the oral route of vaccination in mass prophylaxis against typhoid infection in this country has been stressed by the author (2) in a paper in 1939. But the cost of imported oral vaccines is undoubtedly prohibitive to be utilised for mass prophylaxis. Therefore, apart from the academic value,

*This work was carried out with a Fellowship under the Indian Research Fund Association.

it would also be interesting to analyse the oral vaccines on immunological background and find out the possibilities of producing potent and efficient oral vaccine at economical rates.

EXPERIMENTAL

Immunizing Antigens :

Oral vaccines used for prophylactic and curative purposes essentially consist of dead bacteria and various methods adopted for preparation and preservation of typhoid vaccines have got unequal effects on the potency of the different antigenic constituents of Bact. typhosum. Hence several methods were utilised for the preparation of the antigens.

Two principal forms of antigens were used. One consisted of bacterial emulsion, while the other consisted of water soluble filtrates of the product obtained on lysis of the bacteria. As it can be understood that, while the antigens in the form of bacterial emulsion can easily escape from full action of any reagent under cover of bacterial cell, the watery solution of the antigens of Bact. typhosum will be much more readily acted upon by such reagents, if the latter has really any action on those antigens. It was, therefore, decided to use both the forms of antigens for stability tests.

A. *Bacterial lysate—Endotoxin of Grasset (3) :*

1. *For H and O antigens*: Strain H 901 was grown in Roux bottles and emulsions were made in sterile distilled water. It was then centrifuged, washed with distilled water and resuspended in distilled water in strength of 10,000 million per c.c. It was then subjected to alternate freezing and thawing for 6 times, after which it was filtered through candles. The clear watery filtrate after sterility test was kept in refrigerator. It was found that the average lethal dose of this filtrate for a rabbit weighing one kilogram was between 0.5 cc. to 1 cc. When a sub-lethal dose was injected in rabbits, they gave rise to high titre of H and O antibodies in their sera, showing thereby that this filtrate contained potent H and O antigens.

2. *For Vi antigen*: Attempts were made to extract soluble Vi antigen from filtered lysate of the strain Vi I of Bhatnagar (4), which is rich in Vi antigen and contains a small amount of O antigen but no H antigen. Grasset (5) failed to obtain Vi antigen in the bacteria-free lysate of Vi strains of Bact. typhosum. But Rauss (6) by alternate freezing and thawing could extract an alcohol-precipitable carbohydrate, which stimulated production of Vi agglutinins. In connection with the present series of experiments it was found that filtrates obtained after freezing and thawing of Vi I strain contained Vi antigen only in trace, just sufficient to give rise to a low titre of Vi agglutinins in the serum of rabbits, when the latter received repeated doses of large quantities of bacterial lysate.

Owing to the insufficient quantity of the Vi antigen present in the lysate and failure of rabbits to respond properly to produce Vi antibodies in sufficient titre by injections of these lysates, the latter could not be utilised in experiments for the investigation of stability test of Vi antigen.

B. *Bacterial Emulsions :*

1. *H* plus *O antigens*: As heat is known to have a deteriorating effect on H antigen of Bact. typhosum, formalin was used for killing and preserving H antigen. Emulsion from growth on agar media of H 901 was made in saline containing 0.2% formalin. It was left at room temperature for 3 days. When the emulsion was found sterile, it was kept in refrigerator for future use.

2. *O antigen*: 33% alcoholic extract of O 901 strain was used for O antigen.

3. *Vi antigen*: It is known that most of the common laboratory methods used for preparation of bacterial vaccines have got unfavourable action on Vi antigen of Bact. typhosum. The Vi antigen of Bact. typhosum is destroyed by heat at 60°C for 1 hour (7) as also by preservation in 0.5% carbolic (8). Addition of 0.2% formalin as preservative alters the antigen to such an extent that when rabbits are immunized with such antigen, Vi agglutinins are produced in their sera, but the latter fail to protect mice effectively against virulent strains of Bact. typhosum. Such antigens are considered as 'functionally deficient' (8). But alcohol has been found to be a good bactericide and preservative for Vi antigen (9, 10, 11). For immunization with Vi antigen, therefore, freshly prepared alcohol-killed suspensions were used. For immunizing antigen Vi I strain was mainly utilized because it was rich in Vi antigen and devoid of H and deficient in O antigens.

These antigen preparations were subjected to the action of various reagents such as acid, alkali, peptic-digestion, tryptic-digestion, artificial gastric juice, pancreatic extract, intestinal extract, etc., and then injected into rabbits in order to test the effect of the reagents on the potency of the antigens. If they were found capable of producing antibodies in the sera of rabbits, it would be inferred that the antigens are resistant to the action of the reagents. By thus following the stability of the antigens in the various reactions in the gastro-intestinal tract it would be possible to study the fate of the oral vaccine.

Animal Immunization :

Rabbits were injected intravenously with suitable doses of 'treated' or 'untreated' antigens. After 10 days blood was collected from them and the sera were tested for the presence of different agglutinins and their maximum titres were recorded. Before injections, samples of blood were tested from normal rabbits.

Any rabbit showing agglutination titre of 1 in 50 for O or H antigen and 1 in 20 for Vi antigen before immunization was rejected. It has been observed that while rabbits are highly sensitive animals, which after receiving single injections of H and O antigens of Bact. typhosum would give rise to the production of high titre of O and H antibodies, they are much less sensitive to the antigenic stimulation by the Vi antigen of the same organism. Single administration of comparatively big doses of vaccines either failed to stimulate the formation of Vi agglutinins in their sera or the titre developed was very low. Therefore, injections had to be repeated at intervals of 7 to 10 days in order to ascertain whether the Vi antigen in the 'treated' vaccine remained potent or not.

Agglutination Test :

For H and O agglutination the method described by Felix and Gardner (12) was followed. For Vi agglutination Bhatnagar's method (private communications) by using Vi I strain was principally used, while Watson strain was also included as duplicate control. As natural Vi agglutinin was present in the serum of large percentage of rabbits tested, and rise in the titre of Vi agglutinins after immunization was comparatively low, a control of normal serum of the rabbit was always tested side by side with its immunized serum, when the latter was titrated for Vi agglutinins. Agglutinogen used was living Vi I or Watson strain and readings were taken after incubation for 2 hours at 37°C and for 20 hours at 20°C.

Stability test with each reagent was performed on groups of animals. Owing to individual variation from animal to animal the result naturally varied. This was most marked in experiment with Vi antigen. It has already been mentioned that antibody response to stimulation by Vi antigen is usually small. While some animals in a group receiving a low-potency Vi antigen failed to give rise to Vi agglutinins even after 2-3 doses, others similarly treated showed development of Vi agglutinin in low and moderate titres. In such cases if any rabbit of the injected group showed significant rise in the titre of Vi agglutinin, it was inferred that the injected substance contained potent Vi antigen. The degree of potency was to be judged by comparing this group with the control group, which received the antigen in untreated form.

RESULTS

1. *Stability of the antigens of Bact. typhosum in acid :*

The optimum acidity of the human gastric juice is 0.2% and the duration of food staying in the stomach is about 2 hours. Therefore in this series of tests for acid resistance of typhoid antigens, the strength of the acid was up to 0.2% of hydrochloric acid and period of contact was 2 hours at 37°C. Special attention was devoted to study the effect of various reactions of gastric digestion on the

Vi antigen. Therefore, in experiments with acid, acid-pepsin and gastric juices, the effect of these reagents on Vi antigen will be recorded separately from that of H and O antigens.

TABLE I.
Acid resistance of Vi antigen.

No. of Experiment.	Antigen used.	Percentage of acid.	Dose injected.	No. of rabbits in the group.	No. of rabbits showing significant rise in titre after injection.	Average Vi titre after injection.
1.	Alcoholic Vi I	*Nil.* (control exp.)	7000×10^6 divided in 3 doses.	2	2	320
2.	,, ,,	0.025	,,	2	2	320
3.	,, ,,	0.050	,,	2	2	120
4.	,, ,,	0.200	,,	3	2	80

It has been recorded by Felix and Pitt (9) that Vi antigen is inactivated after treatment with relatively strong concentration of HCl (5%) at room temperature. But it can be seen from the above Table that Vi antigen when kept in contact with hydrochloric acid for 2 hours could resist its action up to the strength of 0.2%. And although its potency is reduced, it could still give rise to Vi agglutinins in low titre.

(ii) *Acid resistance of O and H antigens :*

For testing stability of O and H antigens in acid, antigens were used both in the form of bacterial emulsion as well as lysates. The result is given in Table II.

TABLE II.
Showing acid resistance of O and H antigens.

No. of Experiment.	Antigen used.	Percentage of HCl.	Dose of injection per rabbit.	No. of rabbits in the group.	No. of rabbits showing significant rise in O titre.	Average titre of O agglutinin.	No. of rabbits showing significant rise in H titre.	Average titre of H agglutinin.
1. (Control)	Formalinised emulsion of H 901	*Nil.*	300×10^6	2	2	3,200	2	3,200
2.	,,	0.2	,,	2	2	3,200	2	800
3. (Control)	Endotoxin Lot I	*Nil.*	0.25 cc.	2	2	4,000	2	4,000
4.	,,	0.2	0.25 cc.	2	2	3,600	*Nil.*	100–*

* – means a negative or no agglutination.

It can be seen from the above experiments that the two rabbits after injection of 0.25 cc. of endotoxin of Bact. typhosum developed both H and O agglutinins in high titre. But the rabbits, which received injections of acid treated endotoxin developed high titre of O agglutinins but failed to show H agglutinins in their serum. It has been our experience in large number of experiments that rabbits are highly sensitive animals, which after injection with moderate dose of potent H or O antigen never failed to develop the corresponding antibodies in their sera, although individual variations from animal to animal in titre of antibody response have always been noted. It was, therefore, very likely that the flagellar antigen in the acid-treated endotoxin was destroyed. To test the sensitiveness of rabbits of the last group to H antigen, 0.5 cc. of the endotoxin (untreated) was then injected. This was followed by a rise in H agglutinin to a titre of 1 in 3,200. It can also be seen in the above Table that although acid treated emulsion of Bact. typhosum gave rise to both H and O agglutinins, the titre of H agglutinin was very low and much lower than that of O agglutinin. It can, therefore, be inferred that while O antigen is stable in 0.2% hydrochloric acid for 2 hours, H antigen in acid-treated emulsion has deteriorated, although not totally destroyed as in acid-treated endotoxin, where the antigens being in solution come in more intimate contact with acid in the mixture.

2. *Stability of the antigens in acid pepsin digestion :*

In this series of experiments pepsin was used as powder. Preliminary test for proteolytic power of pepsin was tried with coagulated egg albumen and acid casein solution. It was found satisfactory. 0.2% hydrochloric acid and 2% pepsin was used in the final mixture. It was then incubated at 37°C for 2 hours.

(i) *Stability of Vi antigen in acid pepsin digestion :*

Washed alcoholic suspension of Vi I was used as antigen. The result is given in Table III.

TABLE III.

Showing effect of acid pepsin digestion on Vi antigen.

No. of Experiment.	Antigen used.	Percentage of HCl.	Percentage of pepsin.	Dose injected per rabbit.	No. of rabbits per group.	No. giving rise to significant Vi titre.	Average titre of Vi-agglutinin.
1.	Alcoholic Vi I	0.2	2	5000×10^6 (in 3 doses)	2	2	100
2.	(Control) ,,	*Nil.*	*Nil.*	5000×10^6 (in 3 doses)	2	2	240

It will be seen that as compared with the control, the rabbit injected with acid-pepsin-treated Vi antigen developed lower titre of Vi agglutinins. But the Vi antigen was still potent after treatment with acid-pepsin mixture for 2 hours. The result is similar to that obtained in the experiment with acid-treated Vi antigen.

(ii) *Stability of H and O antigens in acid-pepsin digestion :*

Both endotoxin and formalinised emulsion were used. The result given in Table IV shows that addition of pepsin to the acid solution has the same action on O antigen as when acid only is used. But in case of flagellar antigen the addition of pepsin has enhanced the deterioration of the H antigen.

TABLE IV.

Showing the effect of Acid-pepsin digestion on O and H antigens.

No. of Experiment.	Antigen used.	Percentage of HCl.	Percentage of pepsin.	Hours of incubation.	Dose injected per rabbit.	No. of rabbits per group.	No. giving rise to significant O titre.	Average titre of O agglutinin.	No. giving rise to significant H titre.	Average titre of H agglutinin.
1.	Formalinised H 901	*Nil.*	*Nil.*	...	300×10^6	2	2	3,200	2	3,200
2.	,,	0.2	2	2	,,	2	2	4,000	*Nil.*	100 –
3.	,,	0.2	2	24	,,	2	2	3,200	*Nil.*	100 –
4.	Endotoxin Lot I	*Nil.*	*Nil.*	...	0.25 cc.	2	2	4,000	2	4,000
5.	,,	0.2	2	2	0.25 cc.	2	2	3,200	*Nil.*	100 –

A portion of the 24 hours' acid-pepsin-digested formalinised H 901 was filtered and 0.25 cc. of the filtrate was injected to rabbit No. 22, which developed an O titre of 1 in 3200 but no H titre.

3. *Stability in gastric juice of rabbit :*

It has been our experience that in rabbits food remain in their stomach for a very long time and the stomach is not empty even after starvation for 48 hours. So it was not possible to bring out gastric juice of rabbit by means of catheter, which became easily chocked by semi-digested food particles. Therefore, gastric contents of rabbits were collected by means of stout pipettes or 5 cc. syringes through gastrotomy opening. The rabbits after starvation for 24 hours were allowed to drink water half-an-hour before operation. The abdomen was opened under local anæsthesia with Procain hydrochlor. The gastric content was drawn out by means of pipette. The water, which the rabbit was allowed to drink before operation, helped to dilute the gastric content which would otherwise have been difficult to be sucked into the pipette. The gastric content was then centrifuged. The supernatant fluid was found to contain high percentage (0.15 to 0.3%) of free hydrochloric and good proteolytic properties for coagulated egg albumen. This was used for gastric juice of rabbits.

(i) *Stability of Vi antigen in gastric juice of rabbits :*

A few drops of concentrated washed alcoholic suspension of Vi I strain of Bact. typhosum were added to a few cc's of gastric juice of rabbits and it was incubated at 37°C for 2 hours. The required amount was then diluted in saline and injected

into rabbits. As usual for experiments with Vi antigen, the injecttions had to be repeated at intervals of seven to ten days and every time a Vi suspension, freshly treated with newly collected gastric juice, was used. The result is given in Table V.

TABLE V.

Showing effects of rabbits' gastric juice on Vi antigen.

No. of Experiment.	Antigen used.	Hours of contact with rabbits' gastric juice.	Dose injected per rabbit.	Number of rabbits per group.	Number showing significant rise in Vi titre.	Average titre of Vi agglutinin.
1.	Alcoholic Vi I.	2	4000×10^6 (in 2 doses)	4	3	53.3
2.	(Control) Alcoholic Vi I.	*Nil.*	4000×10^6 (in 2 doses) ...	2	2	240.0

It will be seen from the above Table that the potency of the Vi antigen is reduced by treatment with rabbits' gastric juice for 2 hours, although it is still capable of stimulating Vi antibody in the serum of rabbits.

(ii) *Stability of H and O antigens in gastric juice of rabbits.*

Both endotoxins and formalinised suspension of H 901 were used in the test. The result is given in Table VI.

TABLE VI.

Showing effect of rabbits' gastric juice on H and O antigens.

No. of Experiment.	Antigen used.	Hours of contact with rabbits' gastric juice.	Dose injected.	Number of rabbits per group.	Number showing significant rise in O titre.	Average titre of O agglutinin.	Number showing significant rise in H titre.	Average titre of H agglutinin.
1.	(Control) Formalinised 8 901	*Nil.*	300×10^6	2	2	3200	2	3200
2.	Formalinised H 901	2	300×10^6	1	1	2400	*Nil.*	100−
3.	Formalinised H 901	20	500×10^6	1	1	6400	*Nil.*	100−
4.	(Control) Endotoxin Lot I	*Nil.*	0.25 cc.	2	2	4000	2	4000
5.	Endotoxin Lot I	20	0.50 cc.	1	1	3200	*Nil.*	100−

The result recorded in the above Table shows that while O antigen is perfectly stable in the gastric juice of rabbits, H antigen is totally destroyed by it.

4. *Effect of gastric digestion of monkey :*

One medium sized healthy Rhesus monkey was selected for the experiment. It was kept on banana diet for one week. Then it was starved for 24 hours and 35 cc. of typhoid emulsion (containing 27,000 million per cc.) of a mixture of Vi I (alcoholic), T. E. D. (alcoholic) and H 901 (heat-killed) was introduced into the stomach by means of a catheter. 3 hours after, 5 cc. of the stomach content was withdrawn.

Analysis of the Gastric Content :

Whitish mucoid, slightly tinged with bile, the clotted bacterial clumps formed uniform emulsion on dilution with distilled water. On microscopic examination, most of the bacteria appeared to be partially digested. Acidity—free HCl 0.054%, total acid 0.11%.

0.05 cc. of the gastric content was injected intravenously into rabbit No. 45. It developed the following titre:

Vi — 40 +
O — 3200 +
H — 100 −

This experiment showed that O and Vi antigens were resistant to the gastric digestion of monkey.

After administration of oral vaccine the monkey was kept only on water for another 24 hours and then normal food was given. The blood of the monkey was titrated at intervals of 3 days. The result is given in Table VII.

TABLE VII.

Agglutinin titre in the serum of monkey after oral vaccine.

Days of Oral vaccine.	Titre of Vi agglutinins.	Titre of O agglutinins.	Titre of H agglutinins.
Before	20−	20−	20−
5th day	20 (±)	20+	20−
8th ,,	20 (±)	40+, 80+	20−
11th ,,	20±	40+, 80±	20−
14th ,,	20+, 40±	40+, 80+	20−
17th ,,	20+, 40±	40+, 80±	20−

It may be noted here that no bile was administered before or with the oral vaccine.

5. *Effect of human gastric juice :*

Gastric juice was collected by means of Ryle's tube both in empty stomach as well as after test meal (oat meal). After preliminary titration for acidity each sample was centrifuged and the supernatant fluid was used. Samples of gastric juice showing absence or low content of HCl were rejected.

(i) *Stability of Vi antigen in the human gastric juice :*

Equal parts of gastric juice collected at the height of digestion and typhoid emulsion were mixed together and then incubated at 37°C. After 2 hours the desired quantity was injected into rabbits.

TABLE VIII.

Showing effect of human gastric juice on Vi antigen.

No. of Experiment.	Antigen used.	Hours of contact with human gastric juice.	Dose injected per rabbit.	Number of rabbits per group.	Number showing significant Vi titre.	Average titre of Vi agglutinin.
1.	Alcoholic Vi I	*Nil.*	2500×10^6 (in 2 doses)	2	2	80
2.	Alcoholic Vi I	2	2500×10^6 (in 2 doses)	2	2	60

It can be seen in the above Table that the effect of human gastric juice on Vi antigen is similar to that of rabbits' gastric juice.

(ii) *Stability of H and O antigens in human gastric juice :*

Both resting gastric juice as well as gastric juice at the height of digestion after test meal were used. The result is given below.

TABLE IX.

Showing effect of human gastric juice on H and O antigens.

No. of Experiment.	Antigen used.	Hours of contact with human gastric juice.	Dose injected per rabbit.	*Number of rabbits per group.*	Number showing significant rise in O titre.	Average titre of O agglutinin.	Number showing significant rise in H titre.	Average titre of H agglutinin.
1.	Formalinised H 901	2	350×10^6	2	2	1600	2	150
2.	,, ,,	20	350×10^6	1	1	1600	*Nil.*	100–
3.	Endotoxin Lot I	2	0.25 cc.	1	1	3200	*Nil.*	100–
4.	,, ,,	20	0.25 cc.	1	1	3200	*Nil.*	100–

It may be seen in the above Table that human gastric juice has got strong destructive action on H antigen but little effect on O antigen of Bact. typhosum.

6. *Stability in alkaline reaction :*

The *p*H in the intestinal tract is alkaline. The maximum *p*H of the intestinal juice is about 8.2. Therefore, *p*H of typhoid emulsion was adjusted to 8.2 and then incubated at 37°C for 20 hours. It was then injected into rabbits. The antigenic property of alkali-treated antigens was similar to that of untreated antigens.

The effect of alkaline reaction on antigens can also be noted in series of experiment for stability in bile and pancreatic and intestinal juices, where the reaction was adjusted on alkaline side.

7. *Stability of antigens in Tryptic digestion :*

As trypsin powder with good enzyme activity was not available, freshly prepared alcoholic extract of pig or bovine pancreas was used for the experiment. Pancreas freed from fat was minced. It was then weighed and mixed with equal part of rectified spirit and 3 parts of water. The mixture was shaken and kept in room temperature for 4 days. The fluid was then siphoned and filtered. The filtrate, which showed good proteolytic activity, was then used for trypsin digestion.

After mixing of equal parts of antigen and pancreatic extract, the *p*H was adjusted to 8-8.2 and incubated at 37°C. For control experiments heat-inactivated pancreatic extract was used in place of active pancreatic extract. Heat inactivation was performed by raising the temperature of the pancreatic extract to the boiling point and then boiling it for 5 minutes.

TABLE X.

Showing stability of antigens in tryptic digestion.

No. of Experiment.	Antigen used.	Pancreatic extract.	Hours of contact with pancreatic extract.	Dose of antigen injected.	Number of rabbits in the group.	Number showing significant rise in Vi titre.	Average Vi titre.	Number showing rise in O titre.	Average O titre.	Number showing rise in H titre.	Average H titre.
1.	Alcoholic Vi I	Fresh	20	6000×10^6 (in 3 doses)	2	2	80				
2.	Formalinised H 901	Fresh	20	500×10^6	2			2	1200	2	200
3.	,,	Heat-inactivated (control)	20	500×10^6	1			1	1600	1	1600
4.	Endotoxin Lot I	Fresh	20	0.25 c.c.	2			2	2400	2	250
5.	,,	Heat-inactivated (control)	20	0.25 cc.	1			1	2400	1	1600

The above experiments show that the enzymes of the pancreatic extract has got some deteriorating effect on the H antigen, which may be due to this antigen being probably of the type of a protein in composition (13). Vi and O antigens are fairly stable in this reagent.

8. *Effect of bile and bile* plus *pancreatic extract :*

Sterilised ox bile was used in strength of 2-2.5%. Alcoholic extract of pancreas was used for tryptic digestion as in the previous experiment.

TABLE XI.

Showing the effect of bile and bile plus *pancreatic extract.*

No. of experiment.	Antigen used.	Pancreatic extract.	Percentage of bile.	Hours of contact.	Dose injected.	Number of rabbits in the group.	Number showing significant Vi titre.	Average Vi titre.	Number showing significant 0 titre.	Average 0 titre.	Number showing significant H titre.	Average H titre.
1.	Alcoholic Vi I	Equal parts	2	20	6000×10^6 (in 3 doses)	2	2	80				
2.	Formalinised H 901	*Nil.*	2.5	20	500×10^6	2			2	3200	2	1600
3.	Formalinised H 901	Equal parts	2.5	20	500×10^6	1			1	3200	2	1600
4.	Endotoxin Lot I	,,	2.5	20	0.25 cc.	2			2	3200	1	800

From the above Table it may be noted that bile did not show any special effect on the antigens of Bact. typhosum either when it was used alone or in combination with pancreatic extract.

9. *Effect of intestinal juice :*

Intestinal juice was collected from rabbits. Rabbits were starved for 24 hours and then food was given. 3-4 hours after food, the abdomen was opened under local anæsthesia and the liquid intestinal content was withdrawn by means of a pipette after incision through the walls of the small intestine. The intestinal content was then filtered and centrifuged. The supernatant fluid was then tested for peptone splitting activity.

Typhoid emulsions were treated with equal parts of intestinal juice at 37°C for 20 hours at *p*H 7.6 under cover of toluol. The result is given below.

TABLE XII.

Showing the effect of intestinal juice.

No. of experiment.	Antigen used.	Intestinal juice.	Hours of contact.	Dose injected.	Number of rabbits in the group.	Number showing significant Vi titre.	Average Vi titre.	Number showing significant 0 titre.	Average 0 titre.	Number showing significant H titre.	Average H titre.
1.	Alcoholic Vi I	Equal parts	48	3000×10^6 (in 3 doses)	2	2	160				
2.	Alcoholic Vi I	*Nil.* (control)	...	3000×10^6 (in 3 doses)	2	2	320				
3.	Formalinised H 901	Equal parts	48	500×10^6	2			2	4800	2	1600

It may be seen from Table XII that intestinal juice alone has got very little action on the antigens of Bact. typhosum.

10. *Effect of combined digestion with gastric, pancreatic and intestinal juices :*

In this series of experiments gastric juice was collected from rabbits as in experiment No. 3. For pancreatic juice alcoholic extract of pig's pancreas was used. Intestinal juice was collected as in the previous experiment. Digestion with gastric juice was allowed to take place for 2 hours. For pancreatic and intestinal digestion 20-48 hours were allotted for each. The result is given in Table XIII.

TABLE XIII.

Showing effects of combined digestion with gastric, pancreatic and intestinal juices.

No. of experiment.	Antigen used.	Hours of digestion with gastric juice.	Hours of digestion with pancreatic juice with bile.	Hours of digestion with intestinal juice.	Dose of injection.	Number of rabbits in the group.	Number showing significant Vi titre.	Average Vi titre.	Number showing significant O titre.	Average O titre.	Number showing significant H titre.	Average H titre.
1.	Alcoholic Vi I	*Nil.*	48	48	3000×10^6 (in 3 doses)	2	2	160				
2.	Formalinised H 901	2	24	*Nil.*	500×10^6	2			2	1600	none	20—
3.	Formalinised H 901	2	20	24	1000×10^6	4			4	1200	none	20—
4.	Formalinised H 901	*Nil.*	20	24	1000×10^6	2			2	1600	2	400

It may be noted from the above Table that intestinal juice has got very little destructive action on the antigens of Bact. typhosum.

Discussion

It is now well-known that of the antibodies corresponding to the three antigens of Bact. typhosum, the H antibodies have no protective value (14, 15, 16), while O antibodies give protection against toxic substances (Endotoxin) of Bact. typhosum and Vi antibodies give protection against living virulent strains (7, 8, 17) of the same organisms. Therefore; from the immunity point of view the Vi and O antigens are most valuable, while H antigen is of little importance.

Although earlier work with oral vaccine recorded the production of agglutinating antibodies in the serum of human subjects as well as other laboratory animals after administration of typhoid vaccine per os., there has been hardly any investigation into the stability and absorption of the different antigens of Bact. typhosum in the gastric intestinal tract and their ability to produce the correspond-

ing type of agglutinins in the blood after absorption in the system. It may be pointed out that the discovery of the Vi antigen of Bact. typhosum is of comparatively recent origin and usual methods of preparation of bacterial vaccine, *e.g.*, heat, phenol, formalin, etc., either destroy the Vi antigen or so alter it as to make it 'functionally deficient'. Little attempt has so far been made to include this antigen in potent form in oral vaccine. Particular interest was, therefore, taken to study the fate of this antigen in oral vaccine.

The oral vaccines after being swallowed first come in contact with the gastric juice. And in order to avoid the deteriorating action of gastric juice, most of the commercial preparations of Bili-vaccines consist of killed and dead bacteria put in enterosol-coated tablets or in capsules. This process of manufacture naturally complicates the preparation of vaccine, to which the comparative high cost of imported Bili-vaccines are due. It has been one of the objects of present investigation to study the possibilities of preparing potent oral vaccines of Bact. typhosum at economical rates in this country. Great attention was, therefore, directed to study the stability of the antigens in gastric digestion. It has been seen in the present series of experiments that while H antigen is most labile to the action of HCl, the acid-pepsin digestion and the gastric juice of rabbits, monkeys and human subjects, O antigen is perfectly stable to such treatment for a long time and the Vi antigen stands intermediate between H and O antigens in their comparative stability to such reactions.

When the vaccine is administered well-diluted with water in empty stomach, it quickly passes down from the stomach into the duodenum owing to the watery nature of the gastric content, and the various antigenic factors of the vaccine come in contact with gastric juice for only a short period of time. Therefore, O and Vi antigens of Bact. typhosum in liquid vaccine are likely to be only very slightly affected in the stomach, although Vi antigen, if included in oral vaccines in capsules, will probably have a comparatively better chance of escaping the action of gastric digestion. As the remaining portion of digestive process in the gastro-intestinal tract has got the same effect on oral vaccine either in liquid form or in capsules, it would be reasonable to suppose that a vaccine which contains potent Vi and O antigens, such as alcohol-killed and alcohol-preserved or dried vaccine or merthiolate killed vaccine preserved in concentrated saline of Rainsford (18), would be superior to the current commercial Bili-vaccines, the existing process of manufacture destroys the most important antigen of Bact. typhosum, *e.g.*, Vi antigen.

While *in-vitro* tests with gastric juice have shown that Vi antigen is relatively stable in gastric digestion, feeding experiment on monkey has shown that Vi agglutinins are produced in their sera when Vi antigen is included in oral vaccine. Similar results have recently been recorded by Felix (11) in human subjects.

As has been mentioned before, Vi antibodies may become 'functionally deficient' by treatment with reagents and for the preparation of a therapeutic Anti-typhoid Vi serum Falix and Bhatnagar (8) have advocated the use of natural

Vi antigen in the form of living bacteria. It was, therefore, necessary to see whether the Vi antibodies formed by injections of 'treated' Vi antigens were 'functionally deficient' or not. These results are recorded in the next part of this paper.

Pijper and Dow (19) and Greenwood, Topley and Wilson (20) noted that after oral typhoid vaccine in human cases only O agglutinins are formed but not H. agglutinins. While others including Moor and Brown (21) and Guarnaccil (22) obtained both H and O agglutinins by such vaccination. The variation in the experimental findings may be explained in the light of the findings of the present series of experiments. It has been seen that acid, acid pepsin and gastric juice destroy H antigen. Furthermore, feeding liquid typhoid vaccine to rabbit or monkey or introduction of such vaccine into the stomach of rabbit by means of catheter through mouth or by a syringe and needle through the abdominal opening did not result in development of H agglutinins in their sera. But flagellar agglutinins were formed when the same vaccine was introduced into the small or large intestine by means of syringe and needle (these experiments are fully described in the next part of this paper). The formation of H antibody or its failure was therefore an indication as to what extent the gastric juice has acted upon the vaccine. It may be mentioned that those who obtained H agglutinins after oral vaccines, used mostly the vaccine in capsules. And although pancreatic and intestinal digestion has got some action on H antigens, it will be shown in the next part of the present paper that sufficient amount of the flageller antigen may get absorbed to produce H antibodies before the antigen may be totally inactivated by such digestion.

The effect of digestion in the intestine on the Vi antigen is slow and less marked, and it is possible that sufficient amount of Vi antigen of oral vaccine may reach the intestinal mucosa in potent form to give rise to local immunity of the 'receptive cells' or may get absorbed into the circulation, although it may not, in many cases, reach the threshold of antigenic stimulation to produce detectable Vi antibody in the serum. It may be remembered that the threshold of antigenic stimulation of Vi antigen of Bact. typhosum is fairly high for laboratory animals, so that even when injected into the criculation in comparatively high doses by single injection, they often fail to stimulate production of Vi antibodies in detectable titre.

The O antigen of Bact. typhosum being most stable in the process of digestion in the gastro-intestinal tract, O antibodies are most frequently found in the sera after oral vaccines of Bact. typhosum.

It may be remembered in connection with the present series of experiments, that it is not possible to repeat in test tubes to any great degree of accuracy the most detailed process of digestion of oral vaccine as it takes place in the gastro-intestinal tract of an animal, although indications and information of much value may easily be gathered by *in-vitro* experiments. The tests on the stability and absorption of typhoid antigens are, therefore, also repeated by *in-vivo* methods. These results are recorded in a subsequent part of this paper.

SUMMARY

1. Of the antigens of the oral vaccine of Bact. typhosum, the H antigen is most labile to the process of digestion in the gastro-intestinal tract, O antigen is most stable under such reactions, while Vi antigen stands intermediate between the two.

2. Vi antigen if included in typhoid oral vaccine may be expected to reach the intestinal mucosa in potent form to give rise to corresponding antibodies, whether local or general.

3. In the process of preparation of oral typhoid vaccine, attempts should be made to incorporate the Vi antigen in potent form either in liquid state or in capsules.

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THE EFFECT OF THERMAL TREATMENT AND HYDROGENATION ON THE ABSORPTION OF A FEW VEGETABLE OILS

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The comparative rates of absorption of different fats from the intestinal loops of rats has been reported by Steenbock *et al* (1). They found that certain fats are more rapidly absorbed than others and have also observed the effect thereon of many different substances. The absorption of different oils which have been thermally treated have recieved scant attention as yet. Data on the absorption of a few vegetable oils, normal, thermally treated, hydrogenated cocoanut oil and also of groundnut oil which has been subjected to different degrees of hydrogenation are presented in this communication.

Experimental

The absorption of oils by normal adult rats has been evaluated by balance method. The animals were at first maintained on the following fat free diet, 20 g. of which were given daily per a pair of rat, until a constant excretion of fat was obtained in the fæces. 2 cc. of the normal, thermally treated and hydrogenated oils were then mixed with 16 g of the diet, thereby keeping the calorific value

*Lady Tata Memorial Scholar, 1943-44.

of the daily diet approximately constant. The feeding period was again continued until five consecutive readings, taken at intervals of three days, of the fat content of the faeces was found to be the same.

Composition of diet:

Sucrose	36%
Starch	36%
Casein	10%
Egg white	16%
Salt Mixture (Osborn and Mendell)	2%

Vitamin tablets (Complevite) containing vitamins A, B, C and D, were powdered and adequate amounts were added to this diet.

The oils were successively brought to 200°C, 250°C, 275°C and 300°C and maintained at those temperatures for one hour at the normal atmospheric pressure, with the only exception that at 300°C the oils were heated for a period of forty-five minutes only. The absorption of the following oils has been studied: groundnut oil, cocoanut oil, mustard oil, sesame oil, linseed oil and cow and buffalo ghee. All the oils were commercial products. The samples of cow and buffalo ghees were found, on analysis, to be adulterated.

The chemical properties and the percentage of absorption of the normal, thermally treated and hydrogenated oils are collected in Table I.

TABLE I.

Oil.	Temp. °C	Saponification value.	Iodine value.	Acid value.	Percentage of absorption.
Groundnut	Normal	184	83.5	9.6	99.7
	200	184	81.5	7.2	99.7
	250	186.5	83.0	5.3	99.7
	275	190	84.2	2.8	99.7
	300	192.5	76.0	2.5	99.5
Cocoanut	Normal	250	10.0	3.6	98.4
	200	246	10.0	3.4	98.3
	250	246	10.0	1.1	98.3
	275	246	7.4	0.58	96.4
	300	240	6.6	...	91.9
Mustard	Normal	174	100.0	5.4	95.5
	200	172	99.5	3.8	95.2
	250	172	92.6	3.3	95.2
	275	170	87.0	1.8	91.7
	300	166.5	78.0	...	88.7
Sesame	Normal	189	109	13.4	96
	200	189	107	11.2	96
	250	187	96	4.4	95
	275	189	88	1.3	94
	300	190	83	...	92.5

TABLE I.—(*Contd.*)

Oil.	Temp. °C.	Saponification value.	Iodine value.	Acid value.	Percentage of absorption.
Linseed	Normal	192	187	2.6	97
	200	191	174	2.4	95.9
	250	189	144	2.2	94.0
	275	187	136	...	90.8
	300	187	98	...	78.3
Cow Ghee	Normal	197	56.5	1.3	99.2
	200	199	56.7	0.8	98.2
	250	196	50.8	0.5	97.0
	275	199	48.0	...	96.4
	300	199	42.7	...	90.6
Buffalo Ghee	Normal	195	61.0	2.6	98.8
	200	197.5	60.8	2.4	98.6
	250	195	56.7	1.8	97.8
	275	199	50.0	1.8	93.5
	300	197	46.8	...	85.8
Hydrogenated groundnut					
(Soft)	Normal	191	69.5	...	99.5
(Medium)	Normal	195	65.2	...	99.5
(Hard)	Normal	195	61.0	...	99.5
Hydrogenated cocoanut	Normal	255	7.2	...	98.5

It will be seen from the Table I that thermal treatment changes the character of most of the oils, both chemical and physiological, very significantly. The saponification values of the different thermally treated oils deviate but very little from normal. The acid and iodine values are, however, greatly affected. Iodine values of groundnut and cocoanut oils are not altered at 200°C or 250°C, but at 275°C the iodine value of cocoanut oil drops down to 7.4, while the iodine value of groundnut oil is not altered even at this temperature. At 300°C, however, the iodine value of this oil drops to 76, the initial value being, 83.5. Iodine value of linseed oil begins to change at 200°C—the initial value is 187 and at 200°C, it becomes 174—begins to fall more markedly at successive increases of temperatures, until at 300°C, the iodine value stands at 98. The iodine value of sesame and mustard oils and the adulterated samples of ghees begin to fall at 250°C and with gradual rise of temperature, the decreases in iodine values become more marked.

The physical changes noticed were a change of colour and viscosity. With the exception of the two ghees, the oils assume a reddish brown tinge. There is a gradual rise in the viscosity of the oils with the increase of temperature ; linseed oil when treated to 300°C becomes almost solid at room temperature. The viscosity of ground-nut oil changes very slightly when compared with the other oils.

Thermal treatment up to a temperature 250°C does not affect absorption of the different oils, with the exception of linseed oil. But treatment at 275°C and 300°C lowers the absorption of all the oils, excepting groundnut oil. The percentage of decrease in absorption due to thermal treatment is, however, not uniform. The absorption of cocoanut and sesame oil is slightly lowered, that of mustard oil and adultérated ghees is more marked. The reduction in absorption of linseed oil due to thermal treatment is, however, the most marked. The absorption of this oil, unlike all others, begins to fall when this oil is treated to 250°C. Groundnut oil, however, behaves very uniquely ; its absorption is not at all affected by thermal treatment.

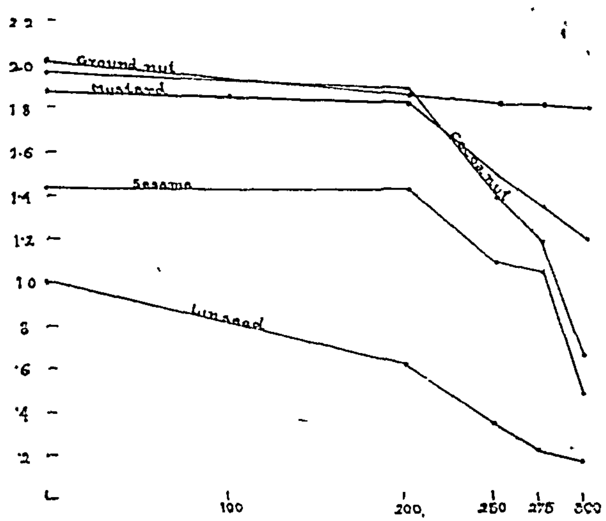

Fig. 1.

Abscissæ = temperature in centigrades.
Ordinate = cc. of N/10 free acid liberated in one hour per every cc. of the incubating mixture.

Hydrogenation of groundnut and cocoanut oils to different degrees does not either affect the absorption or the saponification values of these oils—it is characterised by a gradual fall of iodine value. It is evident, therefore, that the degree of unsaturation does not affect the absorption of oils. This observation also confirms the finding of Peretti and Reale (2).

The study of the rate of hydrolysis of normal and thermally treated oils by lipase *in vitro*, is however quite interesting (Table II).

A mixture of the following composition was incubated at room temperature. 1 cc. of it was withdrawn at zero and one hour and delivered into Erlenmayer flasks containing alcohol-ether mixture. The flasks were then kept for five minutes in a boiling water-bath, cooled and titrated with 0.05 N-alcoholic potash to tritrate the free acidity of the mixture, (fig I).

TABLE II.

Composition of the incubating mixture:

0.1 g. Lipase (Frænkel and Landau)	...	in 1 cc. water.
0.05 g. Na-Taurocholate (Difco)	...	in 1 cc. ,,
5% *Albumin*	...	*0.5 cc.*
2% $CaCl_2$	...	0.5 cc.
Glycine-NaOH buffer (pH 8.8) ...	...	1.0 cc.
Oil	...	1.0 cc.
Total volume	...	5.0 cc.

Oil.	Temp. °C.	Amount of fatty acid liberated in one hour per every cubic centimetre.
Groundnut	Normal	2.04 cc. N/10 acid
	200	1.87 ,,
	250	1.83 ,,
	275	1.83 ,,
	300	1.81 ,,
Cocoanut	Normal	1.96 ,,
	200	1.89 ,,
	250	1.40 ,,
	275	1.20 ,,
	300	0.68 ,,
Mustard	Normal	1.87 ,,
	200	*1.83* ,,
	250	1.50 ,,
	275	1.36 ,,
	300	1.21 ,,
Sesame	Normal	1.44 ,,
	200	1.44 ,,
	250	1.11 ,,
	275	1.06 ,,
	300	0.51 ,,
Linseed	Normal	1.01 ,,
	200	0.63 ,,
	250	0.36 ,,
	275	0.24 ,,
	300	0.18 ,,

It may be noted from Table II that with the increase of thermal treatment, there is a progressive decline in the rate of hydrolysis of the oils. It was observed that the more viscous oils could not be emulsified to the extent it was possible to emulsify the less viscid oil. It is highly probable that the lowered rate of hydrolysis of the more viscous oil is due to the great difficulty experienced in *emulsifying these samples and that the degree of emulsification depends on the* viscosity of the oils; it is also evident from Tables I and II that there exists a close parallelism between the lowered rate of hydrolysis and decreased absorption of the oils. The rate of hydrolysis of the normal oils is also not uniform and the

present author is of opinion that this difference may be due to the difference in the number of easily hydrolysable linkages present in the oils.

In summing up, it may be remarked, therefore, that the oils which become more viscid when subjected to thermal treatment are emulsified to a lesser extent and this in its turn lowers the rate of hydrolysis and, therefore, their absorption.

Summary

1. The percentage of absorption of a few normal, hydrogenated and thermally treated oils has been evaluated by balance method.

2. A few changes in the chemical and physical properties of the oils brought about by thermal treatment have been studied.

3. The degree of unsaturation does not affect absorption.

4. Excepting groundnut oil the absorption of all other oils decreases when they are subjected to thermal treatment.

5. Evidences have been presented to show that the increase in the viscosity of the oils due to thermal treatment reduces the activity of emulsifying agents, thereby retarding lipase activity and absorption.

I should like to express my thanks to Dr. B. B. Sircar and Mr. P. B. Sen for their kind interest and to the Lady Tata Memorial Trust for the award of a scholarship. Thanks are also due to Tata Oil Mills, Cochin and H. V. M. Co., Bombay for a gift of crude and hydrogenated groundnut and cocoanut oils.

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EFFECT OF STARVATION ON THE ALKALINE PHOSPHATASE ACTIVITY OF TISSUES AND THE EFFECT OF DIETARY FACTORS *ON ITS REGENERATION*

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Bodansky (1) had observed that plasma phosphatase activity was lowered enormously in dogs on continued fasting and that, activity was regenerated when the animals were given a carbohydrate meal. In a recent communication Weil and Russel (2) report that twenty-four hour starvation leads to a complete inactivity of the plasma phosphatase of rats and have further shown that activity could be regenerated on ingestion of fatty meals ; carbohydrates and proteins being totally inactive. They have also demonstrated that a few unsaturated acids only are the active components. Kosmer *et al* (3) have reported an increase of the phosphatase activity of the intestine following a fatty or protein meal. In view of the above statements, it was decided to undertake a detailed investigation on the effect of starvation on the phosphatase content of various tissues and the influence of carbohydrates, fats and proteins on the regeneration of phosphatase.

Pigeons have been used as test animals and these were kept without food for one hundred hours but water was allowed *ad libitum*. Various test meals were introduced directly into the stomach after this period. The animals were sacrificed either after six or twenty-four hours following the test meals. Tissues were ground with washed sea-sand in presence of choloroform-water and were allowed to autolyse overnight. 0.05M-sodium-β-glycerophosphate has been used as the substrate and glycine—NaOH as the buffer. The pH of the reaction mixture was maintained at 9.3. The different solutions were brought to 38°C separately and were then mixed. The mixture consisting of 3 cc. of enzyme preparation, 4 cc. buffer and 3 cc. of substrate, was incubated at a temperature of 38°C and 2 cc. of the mixture were withdrawn at zero and two hours and delivered into test tubes containing 2 cc. of 10% trichloracetic acid, shaken and filtered through filter paper (Whatman 42). The inorganic phosphorus in the filtrate was determined by Fiske and Subbarow's method (4).

*Lady Tata Memorial Scholar, 1943-44.

The tissues studied were serum, intestinal scraping, kidney, liver, brain and bone. The influence of the following dietary factors has been noted ; glucose, glycine, tyrosine, egg white, oleic acid, stearic acid, linseed oil and a mixture containing egg white, linseed oil and glucose. These were selected as representatives of a wide variety of dietary factors. The amount of food was administered at regular intervals in three equal doses when the feeding period was continued for six hours and in eight equal doses when the same was continued for twenty-four hours, the net amount administered is indicated in Table I.

TABLE I.

Dietary factor.	Feeding period.	
	6 hours.	24 hours.
Glucose (25%)	6 cc.	18 cc.
Glycine (10%)	5 ,,	12 ,,
Tyrosine (10%)	5 ,,	12 ,,
Egg white (50%)	6 ,,	18 ,,
Oleic Acid Emulsified	4 ,,	12 ,,
Stearic Acid	4 ,,	12 ,,
Linseed Oil	4 ,,	10 ,,
Egg white (raw) 8 cc. / Linseed Oil 5 ,, / Glucose (25%) 12 ,,	6 ,,	18 ,,

The phosphatase activity of tissues from (1) normal animals, (2) starved animals and (3) animals to which different dietary factors stated above were administered, after starvation, are shown in the Tables II—VIII. Activity has been expressed in mg. of inorganic phosphorus liberated in two hours per g. of wet tissue and in case of serum per cc.

TABLE II.

The phosphatase activity of normal tissues and the effect of starvation thereon.

Tissue.	Normal.	Starvation.	Reduction due to starvation.
Serum	0.218	0.062	73%
Bone	0.84	0.40	52%
Kidney	13.5	4.20	69%
Intestine	10.0	4.80	52%
Liver	0.78	0.51	35%
Brain	0.52	0.39	25%

The phosphatase activity of normal tissues will henceforth be abbreviated to N.P.A. and of those tissues following starvation to S.P.A.

The influence of dietary factors on the phosphatase activity of tissues following starvation. (Tables III—VIII).

TABLE III.

Serum.

N.P.A: 0.218 S.P.A: 0.062 Loss of activity 73%	Phosphatase activity after feeding period.		Variation in activity above (+) or below (−) S.P.A. and N.P.A.			
			6 hrs.		24 hrs.	
Dietary factor.	6 hrs.	24 hrs.	N.P.A.	S.P.A.	N.P.A.	S.P.A.
Glucose	0.070	0.083	13%(+)	68%(−)	34%(+)	62%(−)
Glycine	0.073	0.100	17%(+)	66%(−)	61%(+)	54%(−)
Tyrosine	0.100	0.107	61%(+)	54%(−)	72%(+)	51%(−)
Egg white	0.066	0.129	6%(+)	69%(−)	108%(+)	41%(−)
Oleic Acid	0.110	0.138	77%(+)	49%(−)	123%(+)	35%(−)
Stearic Acid	0.058	0.060	*Nil.*	73%(−)	*Nil.*	73%(−)
Linseed Oil	0.143	0.222	130%(+)	34%(−)	250%(+)	1.8%(+)
Egg white, Linseed oil and Glucose.	0.188	0.273	203%(+)	14%(−)	343%(+)	25%(+)

TABLE IV.

Bone.

N.P.A: 0.84 S.P.A: 0.40 Loss of activity 52%	Phosphatase activity after feeding period.		Variation in activity above (+) or below (−) S.P.A. and N.P.A.			
			6 hrs.		24 hrs.	
Dietary factor.	6 hrs.	24 hrs.	S.P.A.	N.P.A.	S.P.A.	N.P.A.
Glucose	0.42	0.40	*Nil.*	52%(−)	*Nil.*	52%(−)
Glycine	0.38	0.42	*Nil.*	52%(−)	*Nil.*	52%(−)
Tyrosine	0.42	0.48	*Nil.*	52%(−)	20%(+)	43%(−)
Egg white	0.52	0.56	30%(+)	38%(−)	40%(+)	31%(−)
Oleic Acid	0.50	0.54	25%(+)	40%(−)	32%(+)	36%(−)
Stearic Acid	0.36	0.40	*Nil.*	52%(−)	*Nil.*	52%(−)
Linseed Oil	0.48	0.58	20%(+)	45%(−)	40%(+)	31%(−)
Egg white, Linseed Oil and Glucose	0.56	0.68	40%(+)	31%(−)	70%(+)	19%(−)

TABLE V.

Kidney.

N.P.A: 13.5 S.P.A: 4.2 Loss of activity 69%	Phosphatase activity after feeding period.		Variation in activity above (+) or below (−) S.P.A. and N.P.A.			
			6 hrs.		24 hrs.	
Dietary factor.	6 hrs.	24 hrs.	S.P.A	N.P.A.	S.P.A.	N.P.A.
Glucose	4.7	5.0	12%(+)	65%(−)	20%(+)	37%(−)
Glycine	4.5	5.8	7%(+)	67%(−)	14%(+)	14%(−)
Tyrosine	6.0	7.2	43%(+)	55%(−)	71%(+)	47%(−)
Egg white	6.8	10.7	62%(+)	49%(−)	155%(+)	21%(−)
Oleic Acid	5.4	6.4	30%(+)	60%(−)	52%(+)	53%(−)
Stearic Acid	4.2	4.6	*Nil.*	69%(−)	7.5%(+)	66%(−)
Linseed Oil	6.1	9.5	45%(+)	55%(−)	126%(+)	30%(−)
Egg white, Linseed Oil and Glucose	8.0	12.0	90%(+)	41%(−)	187%(+)	11%(−)

TABLE VI.
Intestine

N.P.A: 10 S.P.I: 4.8 Loss of activity 52%	Phosphatase activity after feeding period.		Variation in activity above (+) or below (–) S.P.A. and N.P.A.			
			6 hrs.		21 hrs.	
Dietary factor.	6 hrs.	24 hrs.	S.P.A.	N.P.A.	S.P.A.	N.P.A.
Glucose	5.6	6.6	17%(+)	44%(–)	37%(+)	31%(–)
Glycine	6.2	6.2	30%(+)	38%(–)	30%(+)	38%(–)
Tyrosine	7.8	7.6	62%(+)	22%(–)	60%(+)	24%(–)
Egg white	8.0	11.0	67%(+)	20%(–)	130%(+)	10%(+)
Oleic Acid	6.4	7.2	33%(+)	36%(–)	50%(+)	28%(–)
Stearic Acid	5.6	6.0	17%(+)	44%(–)	25%(+)	40%(–)
Linseed Oil	6.8	8.2	42%(+)	32%(–)	70%(+)	18%(–)
Egg white, Linseed oil and Glucose	8.4	10.0	75%(+)	16%(–)	110%(+)	*Nil.*

TABLE VII.
Liver.

N.P.A: 0.78 S.P.A: 0.51 Loss of activity 35%	Phosphatase activity after feeding period.		Variation in activity above (+) or below (–) S.P.A. and N.P.A.			
			6 hrs.		24 hrs.	
	6 hrs.	24 hrs.	S.P.A.	S.P.A.	N.P.A.	N.P.A.
Glucose	0.50	0.60	*Nil.*	35%(–)	18%(+)	20%(–)
Glycine	0.53	0.58	*Nil.*	35%(–)	14%(+)	27%(–)
Tyrosine	0.48	0.50	*Nil.*	35%(–)	*Nil.*	35%(–)
Egg white	0.50	0.58	*Nil.*	35%(–)	14%(+)	27%(–)
Oleic Acid	0.50	0.52	*Nil.*	35%(–)	*Nil.*	35%(–)
Stearic Acid	0.48	0.48	*Nil.*	35%(–)	*Nil.*	35%(–)
Linseed Oil	0.51	0.56	*Nil.*	35%(–)	10%(+)	30%(–)
Egg white, Linseed Oil and Glucose	0.52	0.58	*Nil.*	35%(–)	14%(+)	27%(–)

TABLE VIII.
Brain.

N.P.A: 0.52 S.P.A: 0.39 Loss of activity 25%	Phosphatase activity after feeding period.		Variation in activity above (+) or below (–) S.P.A. and N.P.A.			
			6 hrs.		24 hrs.	
Dietary factor.	6 hrs.	24 hrs.	N.P.A.	N.P.A.	S.P.A.	S.P.A.
Glucose	0.40	0.48	*Nil.*	25%(–)	23%(+)	7%(–)
Glycine	0.38	0.40	*Nil.*	25%(–)	*Nil.*	25%(–)
Tyrosine	0.38	0.39	*Nil.*	25%(–)	*Nil.*	25%(–)
Egg white	0.39	0.41	*Nil.*	25%(–)	*Nil.*	25%(–)
Oleic Acid	0.39	0.40	*Nil.*	25%(–)	*Nil.*	25%(–)
Stearic Acid	0.38	0.38	*Nil.*	25%(–)	*Nil.*	25%(–)
Linseed Oil	0.39	0.40	*Nil.*	25%(–)	*Nil.*	25%(–)
Egg white, Linseed Oil and Glucose	0.40	0.46	*Nil.*	25%(–)	18%(+)	12%(–)

Discussion

Starvation reduces very markedly the phosphatase activity of the different tissues—the maximum reduction takes place in serum, followed by kidney, intestine and bone, liver and lastly brain (Table II). The present investigation reveals that excepting stearic acid all the other components are active in increasing the phosphatase activity of serum (Table III).

The influence of glucose, glycine and egg white, when administered for a period of six hours is slight when compared with the influence of the other components fed for the same period. Oleic acid, tyrosine and linseed oil show remarkable activity but most remarkable is the response when the mixture containing egg white, linseed oil and glucose, (henceforth designated as EWLOG), was given for six hours. The phosphatase activity of serum returns almost to normal—it was only 14% below normal. The influences exerted by the dietary components are revealed more significantly when these were fed for a period of twenty-four hours. Stearic acid is still inactive. Glucose and glycine induce a further regeneration of phosphatase activity, while that due to tyrosine is the same when given for six hours. Egg white which was very slightly active when fed for six hours, exerts its activity more strikingly in twenty-four hours.

Administration of linseed oil alone for twenty-four hours brings a complete regeneration of phosphatase activity to its normal level and the mixture EWLOG stimulates the activity beyond the normal—a 25% increase above the normal level has been noted. In causing a regeneration of phosphatase of bone, glucose, glycine and stearic acid are found to be completely inactive when fed for a period of six or twenty-four hours. Tyrosine which is inactive when given for six hours, causes a 20% rise, if fed for twenty-four hours. Egg white, oleic acid, linseed oil and the mixture EWLOG are fairly active when fed for six hours and their effect becomes more marked if feeding is continued for twenty-four hours (Table IV).

Phosphatase activity of kidney suffers a 69% loss due to starvation (Table V). Stearic acid, again, is inactive. Glucose and glycine are feebly active when fed for twenty-four hours. The other components are fairly active, while EWLOG brings back the activity almost back to its normal level.

It is found, however, in case of intestine (Table VI) that all the components are active in regenerating the losses due to starvation. Tyrosine, egg white and the mixture EWLOG bring about the maximum regeneration in phosphatase activity when fed for a period of six hours. The mixture EWLOG brings back the activity to normal level when given for twenty-four hours and egg white alone when given for the same period stimulates the activity 10% above the normal. Linseed oil and oleic acid also cause a very pronounced increase while that due to tyrosine and glycine is same when these components were fed for six hours.

Liver and brain are the two organs which suffer least due to starvation—decreases of 35% and 25% respectively are found (Table VII and VIII). It has

also been found that the different dietary components are completely inactive, when fed for six hours. Glucose, when given for twenty-four hours, causes 18% increase above the starvation level, in liver ; glycine, egg white, linseed oil and the mixture EWLOG are the other active components in increasing phosphatase activity of liver when fed for twenty-four hours (Table VII). In case of brain, it is noted that glucose and the mixture EWLOG are the only components active in raising the activity of brain phosphatase when these were fed for a period of twenty-four hours (Table VIII).

Weil and Russel (2) have failed to find an increase in plasma phosphotase activity following starvation when pure carbohydrate and protein were given. The present investigation, however, reveals a very consistent increase with all the dietary factors with the single exception of stearic acid. This work, however, confirms their contention that (i) fatty meals are very active in causing a regeneration of plasma phosphatase and (ii) that saturated fatty acids are inactive. But the most remarkable fact, however, is the response when a mixture of egg white, linseed oil and glucose was given for twenty-four hours. This mixture causes the activity to rise beyond the normal level. This mixture has also been found to bring about a very remarkable increase in phosphatase activity of bone, kidney and intestine. It seems that the rise in serum phosphatase activity may be due to the migration of phosphatase from the above tissues to blood.

The response of bone phosphatase to the different dietary factors has a qualitative similiarity to that of serum.

Bellini and Cera (5) have demonstrated an increase of phosphatase in the intestine of the white rat following fat ingestion. Reiser (6) found a rise in inorganic and ester phosphate of the intestinal mucosa of swine during fat absorption. Kosmer *et al* (3) in their investigation on the phosphatase activity of duodenum, jejunum and ileum found the greatest activity after protein and fat meal and least after carbohydrate. This investigation also shows that protein and fatty meals are more active in increasing the phosphatase activity of intestine. It has also been found that of the two amino acids which have been tested, namely, glycine and tyrosine, tyrosine has practically the same influence on the regeneration of phosphatase as egg white, when these two components are fed for a period of six hours following starvation. The influence of glycine is the same when fed for a period of six or twenty-four hours. But at twenty-four hours, the influence exerted by egg white becomes very marked. The comparative values on the regeneration of phosphatase by glycine, tyrosine and egg white when fed for twenty-four hours, following starvation, are respectively as follows: 30%, 60% and 130%. It may be concluded, therefore, from what has been stated above that the various amino acids liberated during the course of digestion of the egg white is responsible for the enormous increase of phosphatase activity of intestine. It would be quite useful to study the effects of individual amino acids on the regeneration of phosphatase activity of intestine.

The influence of the different dietary factors on kidney phosphatase approximately parallels to that of the intestine (Table V & VI).

It has been noted by a number of investigators (7) that the absorption of glucose from the intestine falls very significantly when animals are starved. A very probable explanation that may be deduced from the present investigation to explain the above phenomenon is that due to the pronounced inactivation of the phosphatase system induced by starvation, the whole mechanism of phosphorylation, upon which primarily rests the rate of absorption, is seriously disturbed and hence the lowered rate of absorption of glucose from the intestine of starved animals. Lawroff *et al* (8) have found that absorption of both fat and carbohydrate is increased by previous feeding with proteins and conclude that the diet has influenced some special active process in the absorption. We have already referred to the enormous increase in phosphatase activity of the intestine following a protein meal consisting of egg white only. We are of opinion that this increased phosphatase activity induced by proteins is responsible for the increased absorption of fat and carbohydrate observed by Lawroff *et al.* (8).

Kidney phosphatase plays an important part in the process of reabsorption of sugar from the glomerular filtrate back to general circulation, and therefore consequent to the huge decrease of kidney phosphatase during starvation, ingestion of sugar should lead to glycosuria. Evidences are there to show that such a condition does exist in hunger diabetis. After a prolonged total fasting the capacity for utilisation of ingested sugar is considerably impaired and when sugar is given, it is excreted. The lost ability to utilise glucose is not due to any dysfunction of liver to store glycogen (9). It appears that this condition of hunger diabetis is mainly brought about by the diminution of kidney phosphatase due to starvation.

Glucose is the most potent of all the dietary components in increasing the phosphatase activity of liver and is followed by glycine, egg white and linseed oil—the ketogenic amino acid tyrosine and the two fatty acids are totally inactive. It appears that the dietary principles which promote glucogenesis and glycogenesis would also increase the liver phosphatase after starvation.

Glucose has also been found to be active in increasing the phosphatase activity of brain and the only other potent factor in causing an increase is the mixture EWLOG. Whereas the former increases the activity by 23%, the increase due to the latter is 18%. The latter increase seems also to be due to the glucose component in the mixture, because of the fact that the other components in the mixture when fed singly were quite inactive. Glucose has also been considered to be the most readily available substrate for brain metabolism. The R. Q. of brain *in vivo* reveals it to be approximately unity. Such a respiratory quotient points to the probability, that carbohydrate is the main fuel of brain *in vivo*. Myerson and Halloran (10) and Lennox (11) have also shown that blood on passing through the brain loses carbohydrate. The difference between the glucose contents of arterial and venous blood in brain (9 mg.%) is greater than that between the glucose contents of arterial and venous blood of muscle (arm 5 mg.%, leg 4mg.%). It is interesting to find that glucose also stimulates phosphatase activity of brain after starvation.

It will be noted from what has been discussed so far that the influences exerted by the different dietary factors are not uniform in their behaviour to the different tissues ; carbohydrate has been found to be the potent factor in causing a regeneration of phosphatase in some tissues (brain and liver), while in other, protein or lipoid is the active agent ; evidences have accumulated to show that "Phosphatase" is not a single entity and that it comprises a number of enzymes with distinct substrate specificity (12). It is concluded, from the multifarious nature of the responses, made by tissue phosphatase, to the different dietary factors studied in this investigation, that the nature of the enzyme complex in different tissues differs qualitatively and different constituents which make up the enzyme complex are stimulated in varying degrees by these dietary factors.

SUMMARY

1. Starvation decreases phosphatase activity of tissues ; the magnitude of decrease varies in different tissues.

2. The influences of a number of dietary factors on the regeneration of the phosphatase of different tissues, after starvation, have been studied ; it has been found that the effects of the different dietary components towards the various tissues are not uniform. While some components are active in regenerating phosphatases of some tissues, those same components have been found to be totally inactive towards the others.

3. The influences of the dietary components in most cases, have been found to vary according to the duration of feeding. In some tissues even the active components fail to show activity when feeding is of short duration.

4. The probable relationship between the regeneration of phosphatases and the nature of the substrate and the possibility of the qualitative and quantitative differences of the esterases which constitute the phosphatase complex has been discussed.

Our thanks are due to Dr. B. B. Sarkar, Head of the Department of Physiology, for his kind interest and also to the Lady Tata Memorial Trust for the award of a scholarship to one of us (A.R.)

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ON THE pH OF URINE

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To those interested in the changes which urine may undergo between the time when it is passed and the time when the biochemist or pathologist can deal with it, the following results, calculated in 1944 from experimental observations made in 1938, may be of use.

The urines were of healthy subjects and contained neither protein nor sugar. They were kept in beakers in the laboratory but away from any acid or ammonia vapour, and their pH was found from time to time by Michaelis' titration method (see Cole's Practical Physiological Chemistry), using para nitrophenol and a Gillipsie comparator. Each result is calculated from an average of two or three readings. From a consideration of the variation in these readings it was found that for the same urine at the same time the results could vary by $\pm 3\%$ owing to optical difficulties. For example, if the average reading gave a pH of 6, the divergent ones could give a pH of between 6.18 and 5.82.

In the following tables the same initial represents the same subject.

Specimen.	Time after passing. Hrs.	Mins.	Room temp. °C.	pH	Specimen.	Time after passing. Hrs.	Mins.	Room temp. °C.	pH
B1	0	0	15		F3	0	0	15	
	3	0	15	6.53		0	10	15	7.48
	5	0	15	6.72		2	10	15	7.18
	7	0	15	6.55		4	30	15	7.49
	25	30	15	6.67		23	30	15	7.59
F1	0	0	15			28	40	15	7.62
	0	5	15	6.31		48	50	17	7.85
	1	35	15	6.43		52	5	17	8.01
	25	35	15	6.05					
	27	35	15	6.08					
	47	0	15	6.08					
	71	15	15	6.67					
F2	0	0	17						
	0	10	17	7.23	F4	0	0	17	
	1	20	17	7.28		0	15	17	5.88
	4	10	19	7.46		2	0	17	5.92
	29	10	19.5	7.66		4	35	17	6.03
	47	50	18	7.37		28	45	17	5.89
	52	10	22	7.48		73	15	16	5.93
	72	40	20	7.18		77	15	16	5.78

Specimen.	Time after passing. Hrs.	Mins.	Room temp. °C.	pH
P1	0	0	17	
	0	20	17	7.42
	1	20	17	7.40
	4	10	19	7.71
	28	20	19.5	7.66
	47	20	18	7.50
	53	20	22	7.40
	72	10	21	7.42
P2	0	0	17	
	0	25	17	7.47
	2	10	17	7.21
	4	50	17	7.48
	30	0	17	7.41
	49	25	16	7.16
	53	30	16	7.22
P3	0	0	15.0	
	0	10	15.5	7.31
	3	10	16.5	7.66
	4	25	16.5	7.48
	23	25	15	7.72
	27	55	16.5	8.07
	49	0	15.5	7.50
	52	35	17.5	7.80
	72	45	16	7.42
	75	55	17	7.68
	99	25	17.5	7.68
P4	0	0	15	
	0	10	15	7.12
	1	10	15	7.15
	2	20	16	7.12
	3	10	16	7.12
	4	10	16	7.23
	23	20	13.5	7.08
	27	50	15	7.26
	47	50	13.5	7.35
	71	50	13.5	7.31
	96	50	15	7.48

Specimen.	Time after passing. Hrs.	Mins.	Room temp. °C.	pH
R1	0	0	15	
	0	15	15	6.41
	2	15	15	6.37
	23	15	15	6.46
	27	15	15	6.28
	48	25	15	6.48
R2	0	0	15.5	
	0	5	15.5	5.44
	3	0	16.5	5.37
	4	10	16.5	5.37
	23	30	15	5.35
	27	15	16.5	5.47
	48	25	15	5.39
	52	25	17.5	5.81
	71	20	15.5	5.42
	95	0	15.5	5.42
R3	0	0	15	
	0	5	15	6.46
	2	50	16.5	6.49
	4	15	16.5	6.52
	23	25	15.5	6.58
	28	15	17.5	6.67
	49	0	16	7.21
	52	10	17	6.89
	75	35	17.5	6.69
	99	50	18	6.78
R4	0	0	15.5	
	0	5	15.5	5.21
	1	0	16	5.38
	1	55	16	5.35
	4	0	17	5.32
	5	10	17	5.30
	28	35	17.5	5.41
R5	0	0	15	
	0	10	15	6.52
	1	10	15	6.58
	3	10	16	6.58
	4	10	16	6.55
	23	55	13.5	6.59
	27	55	15	6.64
	48	40	13.5	6.71

Taking F3 as an example: the average pH over two days is 7.60, the maximum and minimum are 7.18 and 8.01, a variation of $\pm 5\%$ from the average, compared with the $\pm 3\%$ for readings taken at the same time. The difficulty of matching colours similarly at different times, with slightly varying daylight, can account for this.

The figures show that the pH of urine kept in an open vessel at temperatures between 13 and 20°C remains constant for 4 days.

Thanks are due to the subjects.

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VITAMIN C AND CARBOHYDRATE METABOLISM. PART IV. THE EFFECT OF VITAMIN C ON THE INSULIN CONTENT OF THE PANCREAS OF GUINEA-PIGS

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(Received for publication, April 4, 1914)

It has been shown previously (1, 2) that the deficiency of vitamin C in guinea-pigs leads to a change in the carbohydrate metabolism as judged by (*a*) glycosuria, (*b*) the diabetic type of the glucose tolerance curve, and (*c*) the depletion of the glycogen content of the liver. It has also been shown that the normal metabolism of carbohydrate is restored after the administration of vitamin C. From the above evidence, it has been suggested that the action of vitamin C is similar to that of insulin. The study of the relation between the vitamin C-status of the body and the insulin content of the pancreas would be useful.

In the present investigation the insulin content of the pancreas has been studied under the following conditions: (*a*) in normal guinea-pigs, (*b*) in scorbutic guinea-pigs, (*c*) in normally fed partially depancreatised guinea-pigs and (*d*) in partially depancreatised guinea-pigs receiving injections of ascorbic acid.

Experimental

Four groups of normal healthy male guinea-pigs of weights varying between 200 g. and 300 g. were taken. The first group consisting of 23 animals was fed on a scorbutic diet for a period of 24 to 28 days until the animals developed scurvy. The second group consisting of 26 animals was fed on a normal diet for two weeks. In the third group, containing 10 guinea-pigs, the animals were partially depancreatised after feeding the animals on a normal diet for two weeks. On the third day after the operation the animals were prepared for the experiment. In the fourth group, which consisted of 10 guinea-pigs, the animals after partial pancreatectomy received 100 mg. of ascorbic acid by injection for three consecutive days.

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Prior to removal of the pancreas, the animals of all the groups were fasted overnight. The animals were stunned by a blow on the head and the pancreases of 5-10 animals were pooled and extracted for insulin according to the method of Best *et al* (3). The crude insulin was dissolved in normal saline of pH 2.5 so that 0.5 cc. of the solution represented 1 g. or 1.4 g. of pancreas. The potency of the solution was tested by studying the hypoglycæmic reaction in rabbits as described by Marks (4). The results are shown in Tables 1-3.

TABLE I.

Normal guinea-pigs.

Expt. No.	No. of guinea-pigs.	Wt. of pancreatic tissue.	Percentage blood sugar reduction by the extract from 1 g. of pancreas.
1	6	6.0 g.	16.0
2	7	7.0	30.5
3	6	6.0	15.6
4	7	5.6	13.0

TABLE II.

Scorbutic guinea-pigs.

Expt. No.	No. of guinea-pigs.	Days on scorbutic diet.	Wt. of pancreatic tissue.	Percentage blood sugar reduction by the extract from 1 g. of pancreas.
1	5	26	5.5	0
2	6	24	6.0	0
3	5	24	5.0	0
4	6	28	6.0	0

TABLE III.

	No. of guinea-pigs.	Weight of pancreatic remnants.	Percentage blood sugar reduction by the extract from 1.4 g. of pancreas.
Partially depancreatised guinea-pigs	10	8.6 g.	0
Partially depancreatised guinea-pigs receiving injections of vitamin C	10	8.9	6.8

After the above experiments, the actual potency of insulin, extracted from the pancreas of scorbutic guinea-pigs, normally fed guinea-pigs and normally fed guinea-pigs receiving injections of 100 mg. of vitamin C for three consecutive days before they were sacrificed, was quantitatively estimated by the rabbit assay method of Marks (4) as described by Burns (5). The results are shown in Table 4.

TABLE IV.

The Insulin content of the pancreas of guinea-pigs.

	No. of guinea-pigs.	Weight of pancreas.	Insulin content per g. of pancreatic tissue. (International unit)
Scorbutic guinea-pigs	21	24.6 g.	0.08
Normally fed guinea-pigs	20	24.0	0.60
Normally fed guinea-pigs injected with vitamin C	18	20.0	0.80

DISCUSSION

The percentage blood sugar reduction in normal rabbits after an injection of 0.5 cc. of insulin extracted from 1 g. of raw pancreas of normally fed guinea-pigs varied from 13.0 to 30.5. The injection of the same volume of insulin extracted from 1 g. of pancreas of scorbutic guinea-pigs, however, gave rise to no reduction of blood sugar. This indicates that the insulin content of the pancreas is greatly diminished in scorbutic guinea-pigs. The average percentage blood sugar reduction in rabbits after the injection of 0.5 cc. insulin extract equivalent to 1.4 g. of pancreatic remnant obtained from the normally fed partially depancreatised guinea-pigs which received injection of heavy doses of vitamin C, was 6.8. The injection of the same volume of insulin extract from the pancreatic remnants of partially depancreatised guinea-pigs receiving no injection of vitamin C gave rise to no reduction of blood sugar. This shows that vitamin C definitely increases the insulin content of the pancreas when the secretion of insulin is diminished as a result of the partial removal of the pancreatic tissue. On assaying the insulin content of the pancreas of guinea-pigs treated in the different way described above and using 18 to 21 animals so that enough insulin could be extracted for the assay it has been observed that the insulin content of the pancreas of scorbutic guinea-pigs is diminished to about $\frac{1}{8}$th of the normal value. The insulin content of the pancreas of guinea-pigs receiving injections of vitamin C does not seem to differ significantly from that of the guinea-pigs fed with normal diet only which is rich in vitamin C. Administration of vitamin C above an optimum level, therefore, does not increase insulin secretion but the absence of vitamin C in the diet produces apparently a striking fall in the insulin content of the pancreas. The glycosuria, the diabetic type of the glucose tolerance curve and the depletion of the liver glycogen as

have been observed with the scorbutic guinea-pigs and partially depancreatised diabetic guinea-pigs may, therefore, be explained by the diminished insulin content of the pancreas in these conditions. The deficiency of vitamin C seems to depress the insulin secretory activity of the islets of Langerhans which possibly leads to the disordered carbohydrate metabolism observed.

Summary

1. The insulin has been extracted from the pancreas of both scorbutic and normal guinea-pigs and also from the pancreatic remnants from partially depancreatised guinea-pigs with or without receiving vitamin C by injection. The potency of the different extracts has been assayed by the study of the percentage blood sugar reduction in rabbits.

2. When 0.5 cc. of the different extracts equivalent to 1 g. of pancreatic tissue is injected into different batches of rabbits, the percentage blood sugar reduction by extracts from the pancreas of normal guinea-pigs varied between 13.0 and 30.6 whereas with the same volume of extracts from the pancreas of scorbutic animals there was practically no reduction.

3. When 0.5 cc. of insulin extract equivalent to 1.4 g. of pancreas is injected into rabbits, the percentage blood sugar reduction by extracts from pancreatic remnants of normally fed partially depancreatised guinea-pigs was 0, whereas with the same volume of extracts from the pancreas of partially depancreatised animals receiving injection of ascorbic acid the percentage blood sugar reduction was 6.8.

4. The insulin content of the pancreas of the scorbutic guinea-pigs is diminished to about $\frac{1}{8}$th of the normal value.

5. The change in the carbohydrate metabolism as observed with scorbutic guinea-pigs is suggested to be due to the diminished insulin content of the pancreas of the scorbutic animals.

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VITAMIN C AND CARBOHYDRATE METABOLISM. PART V. THE EFFECT OF VITAMIN C ON THE HISTOLOGY OF THE PANCREAS OF GUINEA-PIGS

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(Received for publication, April 4, 1944)

It has been shown previously (1, 2) that the insulin content of the pancreas is greatly diminished in scorbutic guinea-pigs. It is, therefore, desirable to study the changes in the islets of Langerhans which are chiefly concerned in the secretion of insulin. A histological study of the pancreas of both the normal and the scorbutic guinea-pigs has, therefore, been made.

EXPERIMENTAL

Two groups of healthy guinea-pigs of weights varying between 163 g. and 420 g. were taken. They were divided into two groups. One of the groups was placed on a scorbutic diet for 24 days and the other was given the normal diet for two weeks. The animals were then selected for the experiment. They were fasted overnight and killed next morning by a blow on the head. A portion from the tail end of the pancreas was fixed in Zenker-formol solution for 24 hours in an ice chest. 7μ-thick paraffin sections were prepared. These were stained with (*a*) Heidenhain's iron hæmatoxylin and (*b*) Heidenhain's 'azan' stains. Every fourth section from the tail end of each pancreas stained with iron hæmatoxylin was studied under the microscope. The number of islets present in each section was counted and the size of the different islets was measured. The results are given in Tables 1 and 2. A statistical analysis of the above results is also presented in Table 3.

*Dr. A. Mitra Research Scholar.

TABLE I.
Normal guinea-pigs.

Animal number.	Number of islets in each section.	Total size of the islets in each section (in square millimetre.)
1	3	75.47
2	6	273.50
3	3	82.50
4	4	62.50
5	11	419.10
6	6	195.65
7	8	279:56
8	12	659.10
9	9	190.87
10	14	496.96
Mean	7.6	273.52

TABLE II.
Scorbutic guinea-pigs.

Animal number.	Number of islets in each section.	Total size of the islets in each section (in square millimetre.)
1	17	2526.52
2	10	385.70
3	10	839.50
4	10	1160.91
5	11	391.50
6	14	1785.21
7	8	1494.59
8	6	418.53
9	18	2514.38
10	8	135.63
Mean	11.2	1168.25

TABLE III.
Statistical analysis.

	Size of islets.	No. of islets.
Difference of the means	894.7	3.6
Standard error of difference	289.16	1.7
t	3.09	2.1
Remarks	highly significant	significant

RESULTS

The sections which were stained with Heidenhain's 'azan' stain were studied to see the different types of cells in the islets of Langerhans. The α-cells were found to be increased in proportion to the β-cells in the islets of Langerhans of the pancreas of scorbutic guinea-pigs. The β-cells were also found to be mostly degranulated in the scorbutic condition. No degenerative changes were observed in the sections of pancreas from both the normal and the scorbutic guinea-pigs.

PLATE I. The photomicrograph of a section of pancreas from a normal guinea-pig.

PLATE II. The same section further magnified.

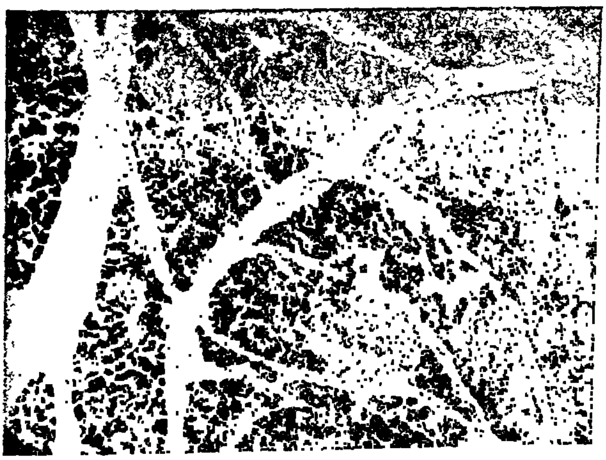

PLATE III. The photomicrograph of a section of pancreas from a scorbutic guinea-pig.

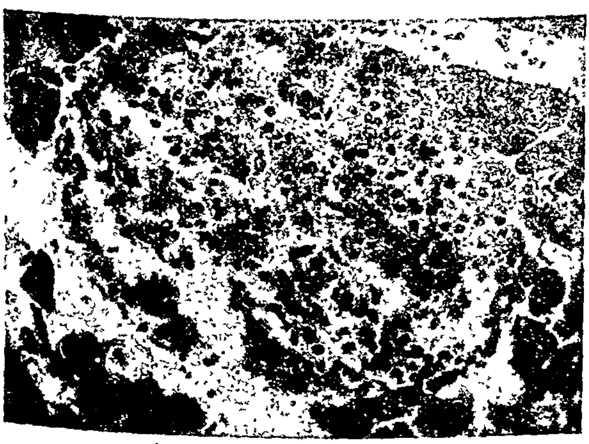

PLATE IV. The photomicrograph of the same section but the magnification is higher.

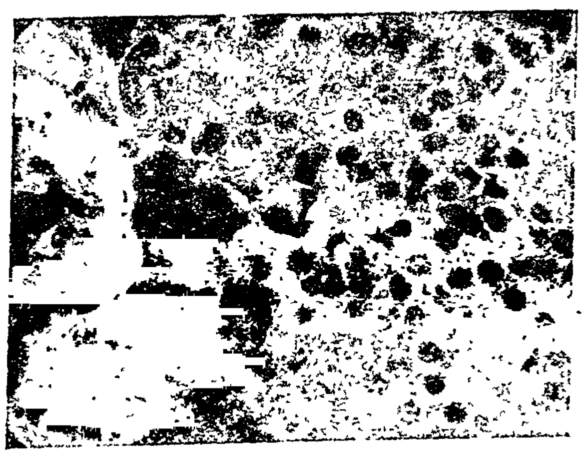

PLATE V. The photomicrograph of a section of pancreas from a scorbutic guinea-pig highly magnified.

Plate I is the photomicrograph of a section of pancreas from a normal guinea-pig. The size of the individual islets of Langerhans is seen to be very small. Plate II, which is the same section further magnified, shows one islet. The β-cells, which are stained black due to the presence of large number of chromatin in them, are found comparatively to be more numerous than the faintly stained α-cells. Plate III is the photomicrograph of a section of pancreas from a scorbutic guinea-pig. The islands of Langerhans are fairly increased in size and number. Some very small islets are seen in the acinous tissue. Whether the acinous cells are being transformed into islet cells or the islet tissue is being formed from preexisting rudimentary islet tissue is not quite clear. Plate IV is the photomicrograph of the same section as shown in Plate III but the magnification is higher. This shows one islet of Langerhans. The faintly stained α-cells are found to be fairly increased in proportion to the β-cells. Plate V is the photomicrograph of a section of pancreas from a scorbutic guinea-pig highly magnified. This shows degranulated β-cells.

Discussion

From the above tablets it is observed that the number of islets is greatly increased in the pancreas of scorbutic guinea-pigs. The size of the islets is also enormously increased, as is evidenced from the size of the islets measured, in the pancreas of scorbutic guinea-pigs. The β-cells are greatly diminished and degranulated. The increase in the size and the number of islets is suggested to be due to nature's attempt to secrete more insulin necessary for the normal utilisation of carbohydrate. Degenerative changes are not seen in the islets. This seems to be related to the fact that if the scorbutic animals are given supplements of vitamin C normal recovery of the animals takes place.

Summary

The number of islets of Langerhans were enumerated and the size of the islets, in the fourth section of the pancreas from its tail end, was measured in sections of pancreas from both the normal and the scorbutic guinea-pigs.

The average size of islets in scorbutic guinea-pigs was 1168.25 sq. mm. while that from the normal guinea-pigs was 273.52 sq. mm. The number of islets in each section in the scorbutic pancreas was 11.2 as compared to 7.6 in normal pancreas.

The β-cells were degranulated in the pancreas from scorbutic guinea-pigs.

No degenerative changes were obtained in the pancreas of the scorbutic guinea-pigs.

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NICOTINIC ACID CONTENT OF FISH

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Although the nutritive value of fish from Indian waters has been investigated by many workers with respect to their fat, protein, mineral content, as well as vitamins A, B_1 and B_2, comparatively less data have been reported with regard to their nicotinic acid content.

The only work reported being that of Saha (1) from this laboratory, on the nicotinic acid content of fresh water fish commonly found in Bengal, and that of Khoranna *et al.* (2) on the nicotinic acid content of fish available in the coastal waters of Waltair.

The present communication deals with the nicotinic acid content of 13 more varieties of fresh-water and 8 varieties of marine fish commonly available in the Calcutta market between the months of January and March.

EXPERIMENTAL.

The fish were obtained daily from the local market and the muscle tissue which forms the edible portion was removed and finely minced, the pulp thus obtained, being directly used for estimation. The method adopted was that of Swaminathan (3) as modified by him. 50 gm. of the pulp were heated in

*Lady Tata Memorial Scholar, 1943-44.

a beaker with 80 cc. of water for 10 minutes at a temperature of 70-80°C in a water bath. 20 cc. of con. HCl were then added and the heating continued for another 10 minutes. The mixture was then centrifuged and heated in a water-bath for an hour in order to hydrolyse the nicotinamide present in nicotinic acid. In some samples a strong colour developed. In order to remove the colouring matter and the protein derivatives after hydrolysis the extract was cooled and adjusted to pH 5-6 with 50% sodium hydroxide. 5 cc. of normal barium acetate were than added and the precipitate formed, removed on the centrifuge. To the clear centrifugate, 1 cc. of 20% zinc sulphate solution was added. Zinc hydroxide was precipitated by the careful addition of concentrated NaOH with phenolphthalein, as internal indicator, so that the mixture had a pH about 9.5. The precipitate was then centrifuged and the excess of barium present precipitated by the addition of 1 cc. of 5 *N*- H_2SO_4. The precipitate thus formed, was again centrifuged and the clear extract so obtained adjusted to pH 7 by the addition of NaOH. In most cases almost colourless solutions were obtained, but at times a slight yellow colour remained which was allowed for by a 'blank' estimation. The final extract was made up to 100 cc. and the colorimetric estimations carried out in the following manner. Aliquots of the extract (usually 10 or 15 cc.) were measured out in 25 cc. measuring flasks, 1 cc. of aniline was added followed by 4 cc. of freshly prepared solution of cyanogen bromide. The contents were then mixed and allowed to stand for 1 minute. 5 cc. of aniline were then added and the volume made up to 25 cc. with water. The yellow colour formed was compared in a Klett colorimeter against a standard solution of nicotinic acid (usually containing 50μg.) treated in the same manner. The estimation must be done within 10 minutes.

The results obtained are given in Table I *a* and I *b*.

TABLE I*a*.

Nicotinic acid content of fresh-water fish.
(Values are given in mg. per 100 gm. of fresh muscle tissue).

Local name.	Zoological name.	Nicotinic acid.
Banghar	...	1.77
Ban	...	0.94
Folui	*Notopterus notapterus*	0.84
Hilsa (small variety)	*Clupea ilisa*	0.87
Bhetka	*Lates calcifer*	0.73
Pona	...	0.71
Bata	...	0.62
Kalabasu	*Labeo calbasu*	0.59
Khoyra	...	0.46
Puti (very small variety)	...	0.44
Magur (very small variety)	*Clasins batrachus*	0.45
Chingri (very small)	...	0.38
Puti	...	0.32

TABLE Ib.

Nicotinic Acid content of sea-fish.
(Values are given in mg. per 100 gm. of muscle tissue).

Local name.	Zoological name.	Nicotinic acid content.
Pomfret	*Stromateus spp.*	3.1
Surmai	*Cybium Kuhli*	2.7
Alva	*Stromateus niger*	2.5
Sea-Vetki	...	2.16
Chak	...	2.00
Atta	...	2.00
Lady's finger	...	1.60
Bangada	...	1.18

Recovery of added nicotinic acid:—

In order to test whether the method adopted would permit added nicotinic acid to be recovered quantitatively, experiments were carried out with added quantities of nicotinic acid. Results obtained are given in Table II.

TABLE II.

Recovery of added Nicotinic Acid (N.A.)
(Values are given in mg. per 100 gm.)

Name.	Amount N.A. added.	Value with added N.A.	Value without added N.A.	Amount recovered.	% of recovery.
Banghar	0.20	1.94	1.77	0.17	85
Folui	0.20	1.09	0.89	0.20	100
Puti	0.20	0.50	0.32	0.18	90
Kalabasu	0.20	0.76	0.59	0.16	80
Bhetka	0.20	0.88	0.73	0.15	75

Discussion

Results as shown in Table-I*a* indicate that of the 13 varieties of fresh-water fish analysed, Banghar (1.77 mg.) is found to be the richest in nicotinic acid, whilst Puti (0.32 mg.) is the lowest. The values are of the same order as those reported earlier by Saha (1). These results confirm the fact that the fresh water fish commonly eaten in Bengal is a poor source of this vitamin. On the other hand, Kodicek (4) and Khorana *et al.* have both reported higher values for the fish analysed by them. Kodicek gives the value for Salmon, Herring and Cod to be as high as 8.4 mg., 4.0 mg. and 3.0 mg. per 100 gm. respectively.

Whilst among the fish analysed by Khorana *et al.* the highest value is that of Hilsa (4.7 mg.) and the lowest that of Seer (1.9 mg.). From these results it was interesting to analyse some common varieties of sea-fish, available in this city, and to see whether when the same technique is applied to all the samples, the difference in the nicotinic acid content in fresh-water fish and sea-fish still shows a marked variation. The sample of Pomfret analysed had the highest value (3.1 mg.) and Bangada had the lowest value (1.12 mg.). These results indicate that on the whole sea-fish is a richer source of nicotinic acid than river-fish.

Results obtained for the recovery of added nicotinic acid shows that except in one case no quantitative recovery was possible. The lowest recovery obtained was 75%. We find the method to be accurate within these limits on considering the limitations of a visual colorimeter and the fact that the final colour is not stable even when the estimations are done within 10 minutes as recommended by Swaminathan (3).

Summary

Thirteen varieties of fresh-water fish commonly found in the Calcutta market, and eight varieties of sea-fish also found in the city market have been analysed for their nicotinic acid content by the modified method of Swaminathan. Sea-fish is found to be richer in this vitamin. The highest value for the sea-fish is that of Pomfret 3.1 mg. per 100 gm. of muscle tissue. Among the river-fish Banghar, (1.77 mg.) had the highest value.

My best thanks are due to Prof. B. C. Guha for his kind help and advice.

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PHYSICO-CHEMICAL STUDIES ON HÆMOLYSIN. PART I. CRYSTALLINE HÆMOLYSIN (LECITHINASE)

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The toxic activities of venoms of poisonous snakes are extremely complex. Nearly all of them have some neurotoxic action, either central or peripheral; many of them possess powerful hæmagglutinic, hæmolytic and cytolytic activities; some of them contain proteolytic or lipolytic enzymes and frequently enzymes

like phosphatase or choline esterase. In the venoms of Indian cobra, which is the subject of the present studies, neurotoxic and hæmolytic activities are present in all of them. The hæmolytic agent has a destructive action upon red blood cells both *in vivo* and *in vitro*.

The researches of Kyes (1) and those of Delezenne and his colleagues (2) have shown that hæmolysis may be caused by an enzymatic component of snake venoms. The enzyme is capable of splitting lecithin with the formation of a powerful hæmolytic agent. To this enzyme the name 'Phosphatidase' or 'Lecithinase' has been given. The enzyme lecithinase acting on lecithin produces a lysolecithin by the separation of an unsaturated fatty acid from the lecithin molecule. The substance so formed (the choline ester of monostearin-phosphoric acid) possesses strong hæmolytic properties. The appropriate name of lysolecithin is given to that material.

Recently much work has been done on the isolation of the neurotoxic fraction of venoms. Micheel *et al.* (3) have obtained from the venom of Naja Tripudians a neurotoxin containing inorganic matter (probably ZnO). Slotta and Fraenkel-Conrat (4) reported that they have prepared a crystalline protein which contains the neurotoxin and the hæmolysin but not the coagulating principle of Crotalus-t-terrificus venom. Ghosh and De (5) have shown that the neurotoxin of the Crotalus-t-terrificus venom can be partially separated from the hæmolysin and, therefore, the crystalline substance obtained by Slotta and Fraenkel-Conrat is a mixture of at least two proteins.

Very little work has been done on the separation of the hæmolysin fraction from the other constituents of the venom. Amongst the earlier workers, who claimed to have separated hæmolysin from neurotoxin the following might be mentioned. Kyes (6) claimed to have separated hæmolysin from neurotoxin by combining it with lecithin and termed the resulting compound lecithide. But later workers have shown that an active hæmolysing agent (lysolecithin) is formed by the interaction of venom with lecithin, which can hæmolyse in absence of both of them. Flexner and Noguchi (7) separated hæmolysin by adsorption with appropriate cells, but this method is unsuitable since it requires the addition of unknown quantities of protein substances. Dunn (8) observed that Crotalus adamanteus venom acting on cephalin produces intensely hæmolytic substances. He separated an active 'Cephalinase' from Crotalus adamanteus venom with strong hæmolytic but no proteolytic activity. The hæmolytic activity of this fraction was of the same order as that of its cephalinase content. Faust (9), however, claimed that he had separated from cobra venom a nitrogen-free substance called ophiotoxin ($C_{17}H_{26}O_{11}$) which resembled a glucoside in composition. According to him these poisons were not proteins but glucosides belonging to the saponin group of hæmolytic agents. He was of opinion that these glucosides were bound to proteins, forming compound

proteins which acted as specific antigens. Later workers did not confirm Faust's results. Franzi (10) working with venoms of vipera aspis and Crotalus terrificus has advanced a theory that hæmolysis is caused by an inorganic catalyst rather than by an enzyme.

The present investigation was undertaken to separate the hæmolytic fraction of some of the venoms in a pure state. For this purpose venoms of snakes of colubridæ family were selected as they possess considerable hæmolytic activity. The venoms collected from the binocellate and monocellate varieties of Naja Tripudians and also venom from Bungarus Fasciatus have been used.

The separation of hæmolysin from the other constituents of cobra venom has been effected by Ghosh and De (11) and by Ganguly and Malkana (12) by treating the venom solution with sodium chloride. The hæmolysin fraction has been further purified by heating the solution of the precipitate at 86°C and finally by cataphoresis of the solution obtained after heat coagulation. For the same protein content the hæmolysin fraction, so obtained, was four times more active than the crude venom. Recently two methods have been evolved by the author (De, 13) for the purification of the hæmolysin and by both the methods a concentration of eleven times of the hæmolysin fraction has been effected.

As both these methods were complicated and tedious and further there was the possibility of denaturation of hæmolysin during these complicated operations, some other method more suitable for purification was attempted. After much experimentation a successful method has been evolved which is described in detail in this section dealing with the crystallisation of hæmolysin.

EXPERIMENTAL

Measurement of hæmolytic activity.—The method employed for the estimation of the hæmolytic activity of various venoms and of their different fractions has been described in a previous paper (13). The amount in γ of the crude venom which hæmolysed the mixture in 2 hours was taken as one unit of hæmolysin. It was not found convenient to determine the exact moment of complete hæmolysis by the naked eye, so estimations were made with hæmolysin sufficient to cause only partial hæmolysis in a given time, and then determine the amount of hæmoglobin liberated by a colorimeter. For this purpose different amounts of the solution to be tested were set up with known amount of crude venom which contained one unit of hæmolysin. After incubation for one hour the unlysed cells were centrifuged and the hæmoglobin liberated in the different tubes was compared colorimetrically with the standard containing the hæmoglobin liberated by one unit of hæmolysin.

TABLE I.

Hæmolytic activity of different venoms.

Venom.	One unit of hæmolysin contained in	1 mg. of the venom contains
Naja Tripudians (variety monocellate)	5.2γ	192 units of hæmolysin
Naja Tripudians (variety binocellate)	4.8γ	282 units of hæmolysin
Bungarus Fasciatus	15.0γ	66 units of hæmolysin

Crystallisation of the Hæmolysin from Naja Tripudians (variety monocellate) Venom.

2 Gm. cobra (monocellate variety of Naja Tripudians) venom were dissolved in 200 cc. of water and the reaction of the solution was adjusted to pH 2.8-3.0 by the addition of 3 *N*-sulphuric acid. To this solution were gradually added with stirring 30 gm. of sodium chloride and the mixture kept in the thermostat at 37°C for 20 minutes. The mixture was filtered using No. 50 Whatman filter paper. The filtrate contained about 5 per cent hæmolysin. The precipitate which contained the major portion of hæmolysin was redissolved in 200 cc. of water. After adjusting the reaction of the solution at pH 4.0-4.2, 20 gm. of sodium chloride were gradually added to precipitate some of the inactive proteins of the venom. The mixture was kept at 37°C as before, and centrifuged to separate the inactive precipitate, the supernatant liquid which contained the active principle was adjusted to pH 2.8-3.0 and the active principle precipitated by the further addition of 8 gm. of sodium chloride. The mixture was incubated as before, filtered under suction to remove the maximum amount of filtrate. The precipitate which contained about 90 per cent of hæmolysin was subjected to fractionation with ammonium sulphate. For this purpose the precipitate was dissolved in 40 cc. water and the solution adjusted to pH 2.8-3.0 and precipitated by the gradual addition of 10.4 gm. of ammonium sulphate. The precipitate which contained the major portion of the hæmolysin fraction was filtered off. This fraction contained some inactive proteins which can be removed by adsorption on Alumina Cγ [Willstätter *et al.* (14)] from a faintly acid solution. For this purpose the precipitate was dissolved in 40 cc. of 2 M acetate buffer of pH 5.6 and treated with 10 cc. (Al_2O_3=396 mg.) suspension of Alumina Cγ and shaken for 30 minutes in the shaker. The alumina was separated from the supernatant liquid by centrifuging. The total volume collected was measured and to this solution solid ammonium sulphate was added until it reached 0.4 saturation. The precipitate formed was removed by filtration after some time and it was found

to contain very small amount of hæmolysin. To the filtrate more ammonium sulphate was added till it has reached 0.6 saturation with respect to ammonium sulphate. This fraction was found to contain 70.8 mg. protein containing 57 per cent hæmolysin with respect to crude venom.

The material collected from 6 gm. of cobra venom by the above method of purification was dissolved in 12 cc. of 0.2 M acetate buffer of pH 6.0 and cooled to 10°C. To this solution were added gradually and with stirring 2.4 gm. of solid ammonium sulphate. Saturated ammonium sulphate was then added, a few drops at a time, every half an hour until crystallisation occurred, which was detected by the silkiness produced on stirring the solution. The silkiness produced on stirring the crystals proved a much more sensitive test than observation under microscope for deciding whether crystallisation has commenced. The saturated ammonium sulphate was so added as not to complete crystallisation within 6 hours otherwise the crystals grew very tiny making it difficult to centrifuge them out. The solution was allowed to stand overnight at 10°C and next day the needle shaped crystals were centrifuged out. Yield of crystals was 182 mg. and contained hæmolytic activity equivalent to 3.19 gm. of cobra venom. Therefore crystalline hæmolysin, obtained from monocellate variety of Naja Tripudians, is 17.5 times more active than the hæmolysin present in crude venom.

Recrystallisation.

The crystals obtained after pouring off the supernatant liquid were dissolved in 10 cc. of 0.2 *N* acetate buffer of pH 6.0. The solution was somewhat turbid, it was centrifuged until clear and then decanted. The solution was cooled to 10°C and crystallised in the manner described for the first crystallisation. It

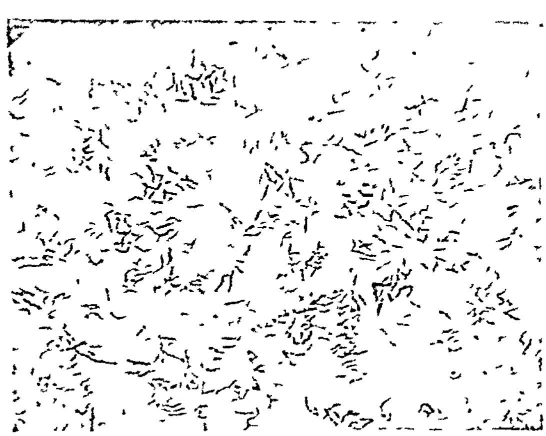

FIG. 1*a*. Crystalline Hæmolysin from Naja Tripudians venom (var monocellate) × 200.

sometimes happens that a portion of the hæmolysin crystals is found to have become insoluble in acetate buffer of pH 6.0. Such crystals have a diminished activity and apparently consist of denatured crystals. This recrystallised product contained 132 mg. protein but no further increase in activity was observed.

TABLE II.

Preparation of crystalline hæmolysin.

Material and manipulation.	per cent protein.	Per cent hæmolytic activity recovery.
2 gm. Naja Tripudians (variety monocellate)	89.26	...
Precipitate obtained from crude venom with 30 gm. NaCl at pH 2.8-3.0	55.14	95.1
Filtrate after precipitation with 20 gm. NaCl at pH 4.0-4.2	26.96	86.6
Precipitate with 8 gm. more NaCl at pH 2.8-3.0	22.13	78.4
Precipitate with ammonium sulphate at pH 2.8-3.0	17.82	75.0
Supernatant liquid after treatment with Alumina Cγ	12.55	68.3
Filtrate with precipitation with ammonium sulphate (0.4 saturation)	4.72	62.8
Final precipitate (0.6 saturation)	3.54	57.0
Crystalline hæmolysin	3.03	53.1

Crystalline Hæmolysin from Naja Tripudians (variety binocellate).

By adopting the above procedure with Naja Tripudians (variety binocellate) venom from a 6 gm. lot, 216 mg. of crystalline hæmolysin were obtained which contained hæmolytic activity equivalent to 3.08 gm. of Naja Tripudians venom, or 70.1 mg. of crystalline hæmolysin contained hæmolytic activity equivalent to 1 gm. of binocellate variety of Naja Tripudians venom. Therefore weight for weight this crystalline hæmolysin is 14.3 times more active than the hæmolysin in crude binocellate variety of Naja Tripudians venom.

Crystalline Hæmolysin from Bungarus Fasciatus Venom.

In the case of Bungarus Fasciatus venom starting from 2 gm. of venom and adopting the same procedure as described before 36 gm. of crystalline hæmolysin (Fig. 1*b*) were obtained containing activity equivalent to 0.96 gm. of crude venom, or 40 mg. of crystalline hæmolysin contained activity equivalent to 1 gm. of crude

Bungarus Fasciatus venom. Therefore, crystalline hæmolysin obtained from Bungarus Fasciatus venom is 25 times more active than its crude venom.

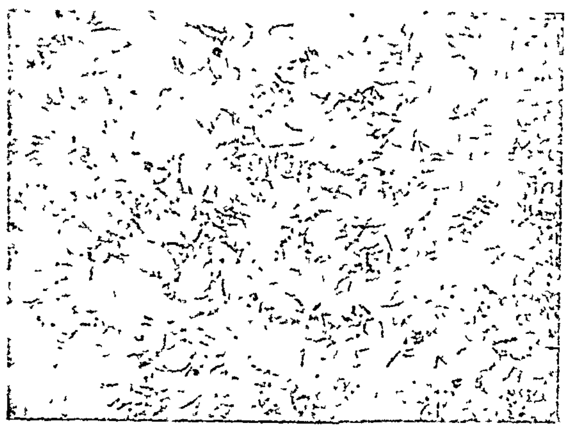

FIG. 1b. Crystalline Hæmolysin from Bungarus Fasciatus venom × 200.

TABLE III.

Hæmolytic activity of different Crystalline Hæmolysins.

Venom.	Purification effected.	1 mg. of the crystalline hæmolysin contains
Naja Tripudians (variety monocellate)	17.5 times	192 × 17.5 = 3360 units of hæmolysin
Naja Tripudians (variety binocellate)	14.3 times	232 × 14.3 = 3318 units of hæmolysin
Bungarus Fasciatus	25 times	66 × 25 = 1650 units of hæmolysin

Preparation of Salt Free Hæmolysin.

Hæmolysin, freed from salts, was used in the subsequent experiments and it was prepared in the following way. After recrystallisation the hæmolysin crystals were centrifuged down and after decanting the supernatant liquid, the paste containing the crystals was placed in a cellophane bag and dialysed at 4°C for 24 hours against frequent changes of cold redistilled water. The solution was taken in a centrifuge tube and any undissolved impurity was removed by centrifuging. The hæmolysin was then precipitated from its solution at 0°C by addition of 4 volumes of ethyl alcohol also at 0°C. The precipitate formed was centrifuged down with frequent cooling and washed with ice-cold ether. The precipitate was then dried in a vacuum desiccator over phosphorus pentoxide. A portion of hæmolysin was dissolved in water and tested with silver nitrate, barium chloride and Nessler's solution, but no indication of chloride; sulphate and ammonium ions was found. This salt-free hæmolysin was used for subsequent experiments.

Toxicity of Crystalline Hæmolysin.

The toxicity of the venoms was tested by intramuscular injection into pigeons of 300 gm. weight and it was found that the m.l.d. for monocellate and binocellate varieties of Naja Tripudians was 0.1 mg. and 0.085 mg. respectively, while in the case of Bungarus Fasciatus venom the m.l.d. was 1.6 mg. But the minimum amount of crystalline hæmolysin required to kill a pigeon of standard weight was 2.4 mg. in the case of the two varieties of Naja Tripudians venom, while the minimum amount of crystalline hæmolysin from Bungarus Fasciatus venom required was 4.0 mg.

Purity Test of Crystalline Hæmolysin.

Although many enzymes have been crystallised, yet the homogeneity of these crystals is still doubtful, as in some cases they have been found to be associated with inert proteins. The individuality of pepsin has been tested by Tiselius *et al.* (15) by electrophoresis in the Tiselius apparatus. It was found that a portion of the dissolved pepsin migrated as an active homogeneous protein but some inactive material was left behind (20-50% of the total). Steinhardt (16) remarked that some crystalline enzymes may be mixtures of protein material. Kunitz (17) observed that beautifully crystalline enzyme preparations may contain large amount of impurities. The solubility test is not in every case a reliable test for homogeneity and careful and varied investigation of each substance must decide the question of its individuality.

A number of tests for the purity of the enzyme have been suggested and carried out:

(*i*) The rate of inactivation compared to formation of denaturated protein.
(*ii*) Sedimentation-rate determinations.
(*iii*) Diffusion measurements.
(*iv*) Electro-cataphoresis.
(*v*) Inactivation by ultra-violet light.
(*vi*) Solubility determinations.

It has been found by Northrop *et al.* (18) that the last method is the only one upon which reliance can be placed since several crystalline preparations which gave constant activity and were homogeneous by other tests, showed the presence of more than one protein when tested by the solubility method. Recently for determining the homogeneity of substances of high molecular weight special methods like ultra-centrifugal sedimentation and electrophoretic measurements in Tiselius apparatus are resorted to. The purity of crystalline hæmolysin has been tested by the solubility method of Kunitz and Northrop (19) and also by the cataphoretic method.

Solubility method.

200 Mg. of crystallised hæmolysin from monocellate variety of Naja Tripudians venom (freed from salt by dialysis) were dissolved in 50 cc. of 0.1 saturated ammonium sulphate, so that 10 cc. of the solution contained 40 mg. of hæmolysin. To each 10 cc. solution were added different volumes of saturated ammonium sulphate solution and water to a total volume of 45 cc. so that the concentration of ammonium sulphate in the different flasks were 0.47, 0.50, 0.54, 0.57 and 0.6. The precipitates formed were centrifuged after one hour. The precipitates obtained were dissolved in water and the protein contents of the different solutions were estimated by determining the total nitrogen and the non-protein nitrogen. The difference between 40 mg. and the protein content in the different precipitates gives the amount of substance in the respective solutions.

On plotting the number of milligram of substance remaining in solution as abscissæ and log. of ammonium sulphate saturation as ordinate a straight line was obtained (Fig. 2) which indicated the purity of the hæmolysin crystals.

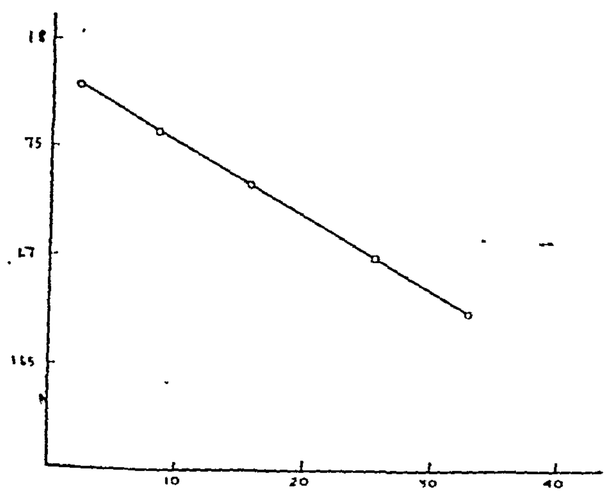

FIG. 2. *Solubility of crystalline hæmolysin in ammonium sulphate solution.*

Abscissæ—mg. Hæmolysin in solution.
Ordinate—log of saturation of ammonium sulphate.

TABLE IV.

Solubility of hæmolysin in ammonium sulphate solution.

Saturation of ammonium sulphate	0.47	0.50	0.54	0.57	0.6
mg. of protein in solution	32.3	24.9	15.2	8.3	2.1

Cataphoretic method.

The apparatus used consists of a four-chambered glass vessel (Fig. 3). The chambers are separated from each other by membranes of suitable material. Finest quality of ultra-fine filter was selected for this purpose. The buffered 10 cc. hæmolysin solution, containing 45 mg. of crystalline hæmolysin (salt free), was

placed in the chamber B and 10 cc. of the same buffer solution were placed in each of the other chambers A, C and D which were put into electrical connection, by means of two agar bridges, with copper sulphate solution contained in two beakers. The copper electrodes were immersed in the copper sulphate solutions.

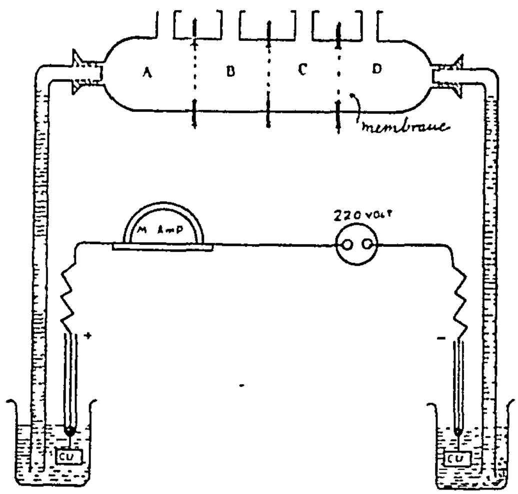

FIG. 3. *Four-chambered cell for cataphoresis of crystalline hæmolysin.*

One of the electrodes was connected directly to one pole of the 220 volt lighting circuit and the other electrode connected to the other pole through a milliammeter. The whole arrangement was placed inside a frigidaire maintained at 4°C. After the passage of 432 coulombs of electricity through the cell, the contents of the chambers C and D were examined for nitrogen and hæmolysin content. Three such experiments were carried out at different pH values, and in no case the ratio of protein to hæmolytic activity varied appreciably from that of the starting solution. Sörenson's phthalate and phosphate buffers were used. The buffer solution was so added that the resultant mixture had an ionic strength of 0.02 M.

TABLE V.

1 mg. of crystalline hæmolysin contained 3360 units of hæmolysin.

pH	Protein content in mg. in chamber.		Units of hæmolysin in chamber.		Units of hæmolysin per mg. of protein in chamber.	
	C	D	C	D	C	D
3.6	3.6	8.6	12000	28000	3333	3250
5.0	3.1	7.2	10200	24200	3293	3361
7.0	2.2	5.5	7400	18800	3363	3418

The slight variation in hæmolytic activity per mg. of protein is within the limits of experimental error. The solubility and the electrocataphoretic experiments, therefore, prove the non-existence of any impurity in the hæmolysin crystals.

Discussion

Crystalline hæmolysin has been obtained from two varieties of Naja Tripudians and Bungarus Fasciatus venom. The activities of the crystalline products are not the same (vide Table III). In the case of monocellate and binocellate varieties of Naja Tripudians venom the hæmolytic activity per mg. of crystalline protein is 3360 and 3318 units of hæmolysin respectively, which may be taken to be equal within the limits of experimental error. Thus the hæmolysins contained in these two types of venoms are probably identical in composition. But in the case of Bungarus Fasciatus venom the activity per gm. of crystalline hæmolysin is only 1650 units, which is one-half of the activity of the crystalline hæmolysin obtained from the two varieties of Naja Tripudians venom. Crystallisation of a particular protein does not always guarantee its absolute purity because in some cases it has been observed that the crystals contain as much as 20 per cent of foreign protein. The above difference in activity suggests that the crystalline product is associated with some inert proteins.

But in the carrier theory it has been postulated that the active enzyme consists of two components, the protein part and the prosthetic group. The protein part is responsible for the specificity and the activity is due to the prosthetic group. It is suggestive that active groups (prosthetic group) present in crystalline hæmolysin from Bungarus Fasciatus venom is one-half of that present in the crystalline products obtained from both varieties of Naja Tripudians venom. In this connection it might be mentioned that Sumner *et al.* (20) prepared three crystalline horse liver catalase preparations which were found to be homogeneous by diffusion but varying in catalase activity from Kat. f. 22,000 to 55,000. They postulated that the catalase activity depends on the number of hematin (prosthetic group) groups in the molecule.

Summary

1. The hæmolysin fraction of venoms of Naja Tripudians and Bungarus Fasciatus species of snakes has been obtained in a crystalline form.

2. The activities of hæmolysin (crystalline) obtained from venoms of monocellate and binocellate varieties of Naja Tripudians have been found to be the same.

3. The activity of hæmolysin (crystalline) from Bungarus Fasciatus venom is one-half of the activity of hæmolysin obtained from both varieties of Naja Tripudians venom.

4. The purity of the crystalline product has been verified by the solubility and cataphoretic methods.

Acknowledgment

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CONTENTS

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THE STABILITY AND ABSORPTION OF THE ANTIGENS OF BACT. TYPHOSUM WITH SPECIAL REFERENCE TO ORAL IMMUNIZATION. PART II.

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(Received for publication, March 29, 1944)

In the first part of this paper (1) the effect of the various digestive processes on the stability of the antigens of Bact. typhosum has been recorded. In the second part of the paper attempts are being made mainly to study the stability and absorption of the antigens in the gastro-intestinal tracts of experimental animals.

EXPERIMENTAL RESULTS

Effects of introducing typhoid antigens into the different parts of the gastro-intestinal tract:

1. *Stomach:* Antigens of Bact. typhosum in the form of killed bacterial emulsion in saline were introduced into the stomach of rabbits by means of a catheter through buccal route after the animal had been starved for 24 hours. Then after a few days' interval blood was taken for testing the formation of antibodies

It may be seen from the following series of experiments that the rise in the titre of agglutinins in the serum of animals, after introduction of the antigens into the gastro-intestinal route, was comparatively slight. In order to minimise the experimental error and also to put definite significance on the rise in the titres, the sera were collected before the administration of the antigens and kept in refrigerator ; they were then tested for normal agglutinins along with the sera collected after the administration of the antigens and their respective titres were recorded.

*This work was carried out with a Fellowship under the Indian Research Fund Association.

TABLE I

Showing the effect of antigens into the stomach.

No. of experiment.	Antigen used.	Dose of antigen.	Type of animal used.	Animal number.	Normal titre.	Result	
						Day after immunization.	Titre.
1.	Formalinised H 901	120×10^9	Rabbit	230	H 20– O 20±	7th day	H 20± O 10+
						15th day	H 20– O 80+
2.	Formalinised H 901, Alcoholic ViI and Bile	96×10^9 18×10^9 0.5 cc.	Rabbit	232	H 20– O 20– Vi 20–	12th day	H 20– O 10+ Vi 20±
						24th day	H 20– O 10+ Vi 20–
3.	Alcoholic Ty2, Alcoholic ViI, *Heat-killed H 901*, and Bile	80×10^9 80×10^9 80×10^9 0.5 cc.	Monkey	XI	Vi 20+ O (20±) H 20–	8th day	Vi 10+, 80± O 10+ H 20–
						15th day	Vi 10+, 80± O 20+ H 20–
4.	Alcoholic T.E.D., Alcoholic ViI and Heat-killed H 901	315×10^9 315×10^9 315×10^9	Monkey	I	Vi 20– O 20– H 20–	5th day	Vi 20± O 20+ H 20–
						8th day	Vi 20± O 80+ H 20–
						11th day	Vi 20± O 10+ H 20–
						14th day	Vi 20+, 40± O 10+ H 20–
						17th day	Vi 20+, 10± O 10+ H 20–

TABLE II

Showing the effect of antigens into the small intestines.

No. of Experiment.	Site of introducing the antigen.	Antigens used.	Dose of antigens.	Type of animal used.	Number of animal.	Normal titre.	Result: Day after immunization.	Result: Titre.
1.	First part of jejunum (near the end of duodenum)	Formalinised H 901 with Bile	80×10^{9} 0.1 cc.	Rabbit	223	H 20– O 20–	7th day 15th day	H 400+ O 100+ H 200+ O 100+
2.	First part of small intestines.	Formalinised H 902 with Bile	80×10^{9} 0.1 cc.	Rabbit	203	H 20– O 20–	7th day 15th day	H 320+ O 200+ H 1600+ O 200+
3.	First part of small intestines.	Formalinised H 901, Alcoholic ViI and Alcoholic Ty_2	10×10^{9} 10×10^{9} 10×10^{9}	Rabbit	231	H 20– O 20– Vi 20–	10th day	H 80+ O 80+ Vi 20–
4.	First part of small intestines.	Formalinised H 901, Alcoholic Ty_2 and Alcoholic ViI	5×10^{9} 5×10^{9} 5×10^{9}	Rabbit	239	H 20– O 20– Vi 20–	10th day	H 20– O 80+ Vi 20–
5.	Middle part of small intestines.	Formalinised H 901 with Bile	80×10^{6} 0.05 cc.	Rabbit	228	H 20– O 20–	7th day 15th day	H 200+ O 400+ H 200+ O 400+
6.	Middle part of small intestines.	Formalinised H 901 and Alcoholic ViI	30×10^{6} 30×10^{6}	Rabbit	229R	H 20– O 20– Vi 20–	8th day	H 320+ O 610+ Vi 20–

It will be seen from table I that none of the animals showed any significant rise in titre of flagellar antibodies in its serum after the typhoid antigens were introduced into the stomach by means of catheter. In all of them there was a significant rise in titre of somatic antibodies. Furthermore, while there was a definite rise in Vi antibody titre in some of the animals, others did not develop significant titre when antigens containing potent Vi antigen were introduced.

2. *Small intestines :* In this series of experiments the antigen was injected into the small intestine. The abdomen of the animal was opened under local anæsthesia with procain hydrochloride. The selected part of the intestine was isolated. The antigen in a small volume of one or two cubic centimetre was injected into the lumen of the intestine by means of a syringe and a fine needle. The abdominal opening was then closed in the usual way.

It will be seen from table II that when typhoid antigen was introduced into different parts of the small intestine, it gave rise to fairly high titre of H agglutinins, excepting in one case. O agglutinins were also found in all cases. The average titre of O antibodies response was higher than when the antigen was introduced into the stomach, although the dose was higher in the latter case. But none of the three rabbits which received Vi antigen into the intestine gave rise to Vi antibodies, while one rabbit after the administration of Vi antigen into the stomach gave rise to a very low Vi titre in its blood. This may be attributed to the comparatively low dose of antigen used in this series of experiments, while the number of rabbits used was only three. The comparative insensitiveness of the rabbits to the stimulation by Vi antigen, individual variation from rabbit to rabbit in antibody producing power, and the high threshold for antigenic stimulation for Vi antibodies may also be taken into consideration. Monkeys appeared to be more sensitive than rabbits to stimulation by Vi antigens administered through parenteral routes.

3. *Large intestines :* The antigens were introduced into the lumen of colon by means of syringe and needle through an abdominal opening as in the case of experiments with small intestine. In experiments for rectal absorption of antigens, vaccines in comparatively bigger doses were introduced high up into the rectum by means of catheter. The result is given in Table III.

It will be seen from Table III that when typhoid antigens with potent Vi, O and H antigens were introduced into the large intestine, either at its proximal or the distal end, the animals gave rise to corresponding Vi, O and H agglutinins.

Sites of absorption of the antigen in the intestinal tract:

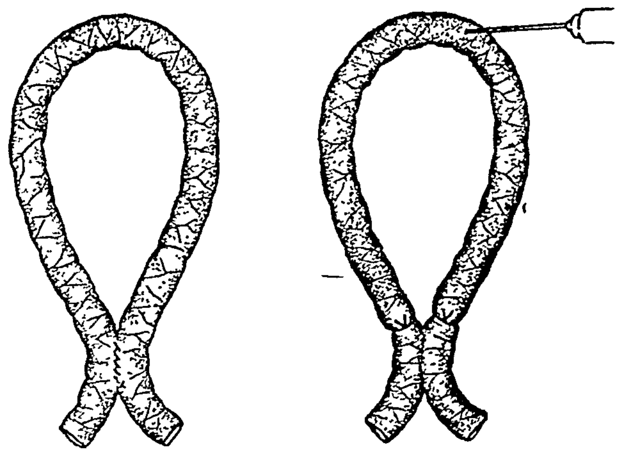
It has been found in the previous experiments that when typhoid antigens were introduced into the proximal or the distal ends of the intestine, they were absorbed into the circulation and gave rise to all the three agglutinins in the serum of the animals. In order to test whether the different antigens of Bact. typhosum are absorbed from the different parts of small intestine, loops of intestines were isolated from the rest of the gastro-intestinal tracts and antigens were introduced into them by a syringe and needle. If the animals developed the corresponding agglutinins in their sera, it could then be inferred that sufficient amount of those antigens had been absorbed from the isolated parts of the intestinal tract.

TABLE III

Showing absorption in the large intestines.

No. of Experiment.	Site of introducing the antigen.	Antigens used.	Dose of antigens.	Type of animal used.	Number of animal.	Normal titre.	Result	
							Day after immunization.	Titre.
1.	Proximal part of	Alcoholic Ty_2	10×10^9	Rabbit	234	Vi 20−	8th day	Vi 80+
	large intestines	Alcoholic ViI and	10×10^9			O 20−		O 40+
		Formalinised H 901	10×10^9			H 20−		H 80+
							13th day	Vi 40+
								O 160+
								H 160+
2.	Per rectum	Formalinised H 901	120×10^9	Rabbit	225	H 20−	7th day	H 20−
		with Bile	0.1 cc.			O 20−		O 100+
							15th day	H 20−
								O 100+
3.	Per rectum	Formalinised H 901 and	9.6×10^9	Rabbit	233	H 20−	12th day	H 40+
		Alcoholic ViI with	4.8×10^9			O 20−		O 20+
		Bile	0.5 cc.			Vi 20±		Vi 80+
							24th day	H 20+
								O 80+
								Vi 40+

Operation:—First stage. Rabbits and monkeys were used. The abdomens of the rabbits were opened under local anæsthesia with procain hydrochloride and on monkeys the operation was performed under a general anæsthesia with chloroform and ether.



First stage. Second stage.

OPERATION OF LATERAL ANASTOMOSIS AND ISOLATION OF A LOOP OF INTESTINES.

Second Stage: After an interval of 15 to 20 days the abdomen of the animal was again opened. The loop of intestines was tied at both ends and the antigen was introduced into the lumen of the loop by a syringe and needle and the abdominal wound was closed with sutures.

In the earlier experiments, when the whole operation was performed in one sitting, the post-operative mortality was very high. Due to the prolonged handling required for the operation of lateral anæstomosis, particularly in the case of rabbits with delicate intestinal walls, some amount of abrasion inevitably took place in the intestinal mucosa, through which the antigens might have leaked into the circulation giving rise to false positive reactions. The possibility of this leackage was eliminated when operation was performed in two stages.

After introducing the antigen into the loop of the intestines the blood of the animal was tested at intervals of 7 to 10 days. If the blood showed development of agglutinins, it could be inferred that the corresponding antigens had been absorbed through that part of the intestine. The result of these experiments is given below. It may be mentioned here that as in the previous experiments, the antigens were washed and resuspended in normal saline before being injected into the loops. The dose of the antigens was made in 1 cc. volume.

It will be seen from table IV that all the three antigens of Bact. typhosum are absorbed from all parts of the intestinal tract. The variation in the results on repetition of the experiments may be due to differences in the condition of the lumen and also of the intestinal walls developed as a result of operation. The digestive juices which would liberate the soluble antigens for their passage through the intestinal mucosa may be deficient as a result of injury during operation. The permeability and absorbing capacity of the intestinal wall might vary from rabbit to rabbit due to the same cause. The variation in the antibody response from

animal to animal must also be taken into consideration, particularly when the antigenic stimulation is small owing to comparatively small doses and slow rate of absorption from a small and comparatively devitalised loop of the intestine.

Functional efficacy of the Vi antibody produced by oral vaccine:

It has been noted that the Vi antibody produced in an animal in response to stimulation by 'natural' Vi antigen (of living bacteria) gives protection against virulent strains of the bacterium. But when the antigen is treated by reagents before injection, it may sometimes give rise to 'functionally deficient' antibodies (2) whose protective power is very poor. It, therefore, remained to be seen, whether Vi antibodies produced by injection of the Vi antigen which had undergone digestion by various reagents were functionally potent or not. It has been shown by Topley *et al* (3), that the Vi antigen can stimulate functionally potent antibodies after proteolytic digestion by trypsin. In the present series of experiments attempts were made to estimate the protective value of the Vi antibodies produced by Vi antigen treated with acid-pepsin, which is equivalent to digestion with gastric juice.

EXPERIMENTAL.

Washed saline suspension of Vi I strain of Bact. typhosum was killed with 75 per cent alcohol. It was then centrifuged, washed with saline and resuspended in normal saline containing 0.2 per cent hydrochloric acid and 4% pepsin. It was then incubated for 2 hours at 37°C., when the antigen was ready for injection. Six doses of the acid-pepsin treated antigen were injected into rabbit No. 247 at intervals of 4 days. Antigens were freshly prepared for each injection and treatment with acid and pepsin was done immediately before injection. Ten days after the last dose, the rabbit was bled ; its blood showed the following titre: Vi 640+, O 640+, H 20−. For control test, rabbit No. 256A was immunized with alcohol-killed suspension, washed in saline. After the administration of 3 doses, each dose containing 1000 million organisms, the rabbit showed the titre of Vi 320+, O 320+. As it is known (4, 5) that Vi antibodies produced by alcohol treated Vi antigen possess full protective value, the serum of rabbit No. 256A was used as a control.

For protection test saline emulsions of 20 hours' living culture of Ty_2 strain were used. The test dose contained in 0.5 cc. volume was injected intraperitoneally. The M.L.D. of Ty_2 was found to be 100×10^6 in this series of experiments. The serum was injected subcutaneously into mice 24 hours before the injection of the test dose of bacteria. The result is given in Table V.

TABLE V

Showing the protective value of Vi antibody produced by acid-pepsin-treated Vi antigen.

No. of Experiment.	Serum No.	Vi titre of Sera.	Dose of Serum.	Test dose.	Number of mice.	Number survived.	Number died.
1.	*Nil.*	...	*Nil.*	100×10^6 (1 M.L.D.)	6	*Nil.*	6
2.	*Nil.*	...	*Nil.*	300×10^6 (3 M.L.D.)	12	*Nil.*	12
3.	247	640	0.4 cc.	300×10^6 (3 M.L.D.)	12	12	*Nil.*
4.	256A	320	0.4 cc.	300×10^6 (3 M.L.D.)	12	8	4

TABLE IV

Showing the absorption of antigens from parts of intestines.

No. of Experiment.	Site of the loops of intestine.	Antigen used.	Dose of antigen.	Type of animal used.	Serial number of animal.	Normal titre.	Result	
							Days after immunization.	Titre.
1.	First part of Jejunum.	Formalinised H 901 and Alcoholic ViI	2×10^9 2×10^9	Rabbit	227	Vi 80+ O 20+ H 20−	10th day	Vi 160+ O 20+ H 20−
2.	Middle part of Jejunum.	Formalinised H 901 and Alcoholic ViI	6×10^9 6×10^9	Rabbit	215B	Vi 20− O 20± H 20−	7th day	Vi 160+ O 40+ H 320+
3.	Middle part of small intestines.	Formalinised H 901 and Alcoholic ViI with Bile	10×10^9 10×10^9 0.1 cc.	Monkey	T5	O 20− H 20−	10th day	O 20+ H 20+
4.	Small intestines.	Formalinised H 901 and Alcoholic ViI	6×10^9 6×10^9	Monkey	T6	Vi 20− O 40+ H 20−	10th day	Vi 320+ O 320+ H 80+
5.	Small intestines just proximal to ileo-cæcal junction.	Formalinised H 901 and Alcoholic ViI	10×10^9 10×10^9	Monkey	T2	Vi 20− O 20+ H 20−	10th day	Vi 20− O 40+ H 160+
6.	Middle part of large intestines.	Formalinised H 901 and Alcoholic ViI	6×10^9 6×10^9	Rabbit	238	Vi 20− O 20− H 20−	7th day 17th day	Vi 40+ O 20+ H 20+ Vi 20+ O 20± H 20−
7.	Middle part of large intestines.	Formalinised H 901 and Alcoholic ViI with Bile	20×10^9 20×10^9 0.1 cc.	Rabbit	236	Vi 20− O 20± H 20−	7th day	Vi 160+ O 320+ H 640+

It can be inferred from the above table that serum No. 247 obtained by immunization with acid-pepsin treated Vi antigen gave solid protection to mice against the living culture of the virulent Ty_2 strain of Bact. typhosum. It is obvious that the protection was not due to the low titre O antibodies, which have very little protective power against a living Vi strain. The protective value of serum No. 247 also stands well when compared with that of No. 256A. Owing to the shortage of mice the protection tests could not be followed any further.

Discussion

It was observed by *in-vitro* tests that H antigen was quickly destroyed by gastric digestion, while it was relatively stable in the process of digestion in the intestinal tract. When the vaccine containing potent H antigen was introduced into the stomach the failure to stimulate the flagellar antibodies was due to the destruction of this antigen by gastric digestion, while sufficient amount of O and Vi antigens escaped destruction in the stomach and got absorbed through the intestinal tract to give rise to a low titre of O and Vi antibodies. But when vaccines were introduced into the small or large intestinal tract, sufficient quantities of H, O and Vi antigens remained unaltered in the process of digestion and were absorbed into the circulation to stimulate definite rise in the titres of the corresponding antibodies.

In absorption and stability tests in the isolated loops of intestine and also by the rectal administration of antigen, it was observed that Vi, O and H antigens were absorbed from all parts of small and large intestines. But the titres of the agglutinins found in all these experiments were very much lower than those obtained when comparatively small doses of the vaccine were injected into the system either intravenously or intramuscularly or subcutaneously. This was possibly due to the slow rate of absorption of the antigens and the gradual deterioration of the antigens in the process of digestion in the gastro-intestinal tract of which the most marked reaction was the destruction of H antigen in the gastric juice. Owing to the progressive destruction of the antigens of oral vaccine and also the slow rate of absorption of its unaltered antigens, the concentration of the latter in the circulation might not have reached the same level as when the antigen was injected by any of the parenteral routes. Consequently the antibody formation in the blood after administration of oral vaccines was at a much lower level.

But if one considers the oral vaccine from the point of view of local as well as general immunity, it may be inferred from the results of the present experiments that the antigens of oral typhoid vaccines have a good chance of reaching the intestinal mucosa in potent form. There they may form a local tissue immunity in the receptive cells and also stimulate a general antibody response in the system of the animal by being absorbed into the circulation through the intestinal mucous membrane. The different antigens of Bact. typhosum have been shown to possess a varying degree of susceptibility to the process of digestion throughout the gastro-intestinal tract. But the antigens, which give rise to the protective antibodies, namely, Vi and O antigens, when given by oral route are fairly stable and very often give rise to a comparatively low titre of corresponding antibodies in the blood.

The rôle of bile in oral vaccine has been investigated by previous workers (6, 7, 8). Besredka believed that bile was essential for successful immunization by

oral vaccines and bile helped in the absorption of antigens by causing denudation in the mucous membrane of the intestine. But Ray and others have demonstrated that bile acts by increasing the permeability of the mucous membrane of the intestine and thereby helps the absorption of the antigens. In the present series of experiments bile was incorporated in some of the experiments, while in others bile was excluded. Although the action of bile was not thoroughly investigated, no particular advantage could be noted in the present experiments by including bile with the antigens.

It has also been shown by mouse-protection tests that the Vi antibodies stimulated by Vi antigen, which had been subjected to gastric digestion, retain full protective value. Therefore the oral typhoid vaccines containing O and Vi antigens may be administered in liquid form with almost the same efficacy as that of vaccines in capsules or enterosol coated tablets.

In the light of the present series of experiments it may be recommended that in all oral typhoid vaccines potent Vi antigen should always be incorporated.

SUMMARY

1. Except in the case of gastric digestion of H antigen, the three antigens of Bact. typhosum are relatively stable in the process of digestion in the gastro-intestinal tract, although all of them are slowly and progressively destroyed in the digestive tract.

2. The rate of absorption of the antigens of Bact. typhosum in unaltered form in the intestinal tract is very slow, although all the three antigens are more or less absorbed throughout the intestinal tract.

3. The titre of antibody formation in the blood of laboratory animals is consequently very low for all the three antigens.

4. The antigens of the oral vaccines consisting of Bact. typhosum are likely to reach the receptive cells of the intestines in potent form and may give rise to local immunity.

5. The Vi antigen after gastric digestion can give rise to Vi antibodies, which are fully potent in mouse protection tests.

6. Vi antigen should always be incorporated in all oral typhoid vaccines.

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VITAMIN C AND CARBOHYDRATE METABOLISM. PART V. THE ADRENALINE AND THE ASCORBIC ACID CONTENTS OF THE ADRENAL GLANDS OF GUINEA-PIGS

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In view of greatly diminished insulin content of the pancreas of the scorbutic guinea-pigs (1, 2) it is necessary to investigate whether the adrenaline of the adrenal gland, which is also concerned in carbohydrate metabolism, is affected in any way in scurvy. The adrenaline and the ascorbic acid contents of adrenal glands have, therefore, been determined in both the normal and the scorbutic guinea-pigs.

Of the different chemical methods for the estimation of adrenaline the use of Folin's method (3) as modified by Barker, Eastland and Evers (4) was found to be suitable. It was pointed out by Guha (5) and by Birch, Harris and Ray (6) that Folin's tungstic acid reagent gives a blue colour with ascorbic acid like adrenaline. Rees(7) observed that the blue colour with Folin's reagent is proportionate to the amount of ascorbic acid present and that if the ascorbic acid in the adrenal gland extracts was first determined by titration with 2:6-dichlorophenol indophenol and the blue colour corresponding to the amount of ascorbic acid present was determined on a sample of standardised ascorbic acid solution, this value subtracted from the total blue value of the sample gave the true adrenaline content of the sample. The value of adrenaline thus determined in adrenal gland extracts was found to correspond to that determined biologically. V. Euler (8) and Schild (9) also showed that no significant difference existed between the pressor activity and the adrenaline content determined colorimetrically in

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adrenal gland extracts, which shows the reliability of the chemical method of Rees (7). In this investigation Rees' modified method for the chemical estimation of adrenaline was, therefore, adopted.

EXPERIMENTAL

Guinea-pigs placed on a scorbutic diet for 22 to 25 days and guinea-pigs fed with normal diet for 15 days were fasted overnight and the adrenal glands were removed next morning after stunning the animal by a blow on the head. The glands were carefully freed from the connective tissue and transferred to a weighed bottle containing normal saline and a few drops of 10% trichloracetic acid. The bottle was again weighed. The adrenaline in the glands was extracted by the method of Barker and Marrian (10) with trichloracetic acid. The adrenaline was determined in an aliquot of the extract by the method of Rees (7) and ascorbic acid was estimated in another aliquot with a standardised solution of 2: 6-dichlorophenol-indophenol. The results are shown in Tables I and II and the statistical analyses of the data are given in Table III.

TABLE I.

Scorbutic guinea-pigs.

No. of animals.	Average wt. per animal. (g.)	Wt. of pooled adrenal. (mg.)	Wt. of adrenal per 100 g. of body weight. (mg.)	Ascorbic acid per 1 g. of adrenal. (mg.)	Adrenaline per 1 g. of adrenal. (μg.)	Adrenaline per pair of adrenals. (μg.)
4	310	1140	91	0.032	859	245
4	374	1120	93	0.038	675	189
6	292	1700	96	0.021	707	200
7	340	2840	119	0.048	636	259
Mean	...	...	100	0.035	719	...

TABLE II.

Normal guinea-pigs.

No. of animals.	Average wt. per animal. (g.)	Wt. of pooled adrenal. (mg.)	Wt. of adrenal per 100 g. of body weight. (mg.)	Ascorbic acid per 1 g. of adrenal. (mg.)	Adrenaline per 1 g. of adrenal. (μg.)	Adrenaline per pair of adrenals. (μg.)
4	311	720	57	0.915	502	90
4	310	860	69	0.732	230	49
4	237	560	59	0.828	459	64
4	323	760	59	1.184	263	50
4	397	980	62	1.351	315	77
Mean	...	...	61.2	1.002	353.8	...

TABLE III.

Statistical analyses.

	Wt. of adrenal per 100 g. of body wt. (g.)	Ascorbic acid per 1 g. of adrenal. (mg.)	Adrenaline per 1 g. of adrenal. (μg.)
Difference of the means	38.8	0.967	365.1
Standard error of difference	6.187	0.13078	74.66
t	6.27	7.39	4.89
Remarks	Highly significant	Highly significant	Highly significant

Discussion

The adrenaline content is found to be significantly increased in the adrenals of scorbutic guinea-pigs. It is a well known fact that the two adrenal glands, namely, adrenal and islets of Langerhans secrete hormones, producing opposite glycæmic effects. Cori and Cori (11) observed that when 0.02 mg. of adrenaline per 100 g. rat is injected subcutaneously blood sugar and blood lactic acid levels rise and liver glycogen values diminish for a short time. Cori and Cori (12) also showed that insulin was inhibitory to hepatic glycogenolysis. The lowered glucose tolerance and the low glycogen content of the liver of scorbutic guinea-pigs might, therefore, be partly due to the action of adrenaline in the absence of the opposing action of insulin.

The ascorbic acid content of the adrenal glands is greatly diminished in scorbutic guinea-pigs. The size of the adrenal gland, however, is significantly increased in scorbutic guinea-pigs. The increase in the secretion of adrenaline is, however, not due only to the increase in size of glands because the adrenaline content per gramme of gland is also significantly increased in scurvy.

The diminished insulin content of the pancreas of the scorbutic guinea-pigs is, therefore, not merely due to the lowered vitality of the tissues of the scorbutic animals. The relation of vitamin C to insulin secretion appears, therefore, to be specific in some degree. It is also interesting to note that in vitamin B_1-deficiency, which also affects carbohydrate metabolism, insulin is not diminished as shown by Best *et al* (13). This would further indicate some specific relation between this particular vitamin, ascrobic acid, and insulin synthesis in the body.

Summary

The adrenaline was determined chemically in the extracts prepared from adrenal glands of both scorbutic and normal guinea-pigs. Vitamin C was also estimated in those extracts.

There is significant increase in the adrenaline content of adrenal glands of scorbutic guinea-pigs though the vitamin C of the adrenal glands is greatly diminished in scurvy as opposed to the decrease in the insulin content of the pancreas. The lowered glucose tolerance and the low glycogen content of the liver of scorbutic guinea-pigs might, therefore, be partly due to the action of adrenaline in the absence of the opposing action of insulin.

It is suggested that the relation of vitamin C to insulin formation in the body is somewhat specific in nature.

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THE EFFECT OF THERMAL TREATMENT ON THE ABSORPTION OF A FEW ANIMAL FATS AND HYDROGENATED GROUNDNUT OIL.

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The absorption of a few vegetable oils and the effect of thermal treatment and hydrogenation thereon has been reported from this laboratory. The present communication deals with the absorption of a few animal fats and hydrogenated groundnut oil.

The percentage of absorption has been evaluated by balance method. The composition of the diet and the method of thermal treatment of the fats were as already reported (1). The chemical properties and the percentage of absorption of the normal and thermally treated oils are given in Table I.

TABLE I.

Oil.	Temp. C	Sap Value.	Iodine Value.	Reichert Value.	Percentage of absorption.
Cow-Ghee	Normal	220	35	20	98
	200	220	35	20	97.6
	250	218	33	18.6	97
	275	220	31.5	17.3	93
	300	216	28.6	16.4	89
Buffalo-Ghee	Normal	227	29.5	26	95
	200	226	26.8	26.2	95
	250	225.8	26.6	24.6	94.6
	275	226	24.2	22.7	90.2
	300	222	21.5	21.6	87.6
Lard	Normal	196	60	...	97
	200	195.3	61.5	...	97
	250	192	60.8	...	96.4
	275	194	59.6	...	92
	300	192.6	56.8	...	90.4
Hydrogenated Groundnut oil	Normal	191.5	66.8	...	97
	200	190	66.8	...	97
	250	190.6	66.0	...	95.2
	275	189.6	65.3	...	93.0
	300	187.5	62.6	...	88.2

*Lady Tata Memorial Scholar, 1943-44.

Discussion.

In the previous communication (1) it was pointed out that the decrease in the percentage of absorption of thermally treated oils was due to the increase of viscosity. In an attempt to ascertain whether the unsaponifiable matter from normal and thermally treated oils affected the rate of lipase activity, a few experiments were undertaken to determine the effect of adding the unsaponifiable matter to the incubating mixture. It was found that the unsaponifiable matter did not influence the rate of hydrolysis of fat.

Summary.

The percentage of absorption of a few animal fats and hydrogenated groundnut oil and the effect of thermal treatment on absorption and chemical properties of the above oils has been reported.

My thanks are due to Dr. B. B. Sircar and Mr. P. B. Sen of the Dept. of Physiology for their kind interest and to the Lady Tata Memorial Trust for a personal grant. My thanks are also due to Messrs. H. V. M. & Co., Bombay for the supply of hydrogenated oils.

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ALKALINE PHOSPHATASE ACTIVITY IN EXPERIMENTAL SCURVY.

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The influence of ascorbic acid on the phosphatase activity has been the subject of many interesting studies. In infantile scurvy the serum phosphatase is reduced and rises after the administration of ascorbic acid (1). Alkaline serum phosphatase activity has been shown to be diminished in guinea-pigs, receiving a scorbutogenic diet. It has also been demonstrated that supplements of ascorbic acid to the diet produce an increase in phosphatase activity (2).

It was believed that in experimental and natural scurvy there was no quantitative diminution of phosphatase and that ascorbic acid in some way activates the phosphatase system (3). This belief was, however, discarded when it was shown by King and Delory (4) that the hydrolysis of the phosphoric esters commonly used for phosphatase determination by serum phosphatase was not influenced by ascorbic acid *in vitro* ; though *in vivo* experiments it has been found that withdrawal of ascorbic acid from the diet results in a lowering of serum alkaline phosphatase activity (5).

*Lady Tata Memorial Scholar.

The investigation under report was undertaken in an attempt to study the behaviour of alkaline phosphatase of different tissues of the same animal in experimental scurvy.

Experimental.

Two groups of healthy and normal guinea-pigs weighing 240-265 g. in weight have been used. The first group was fed on green grass and germinated gram while the second was kept on a scorbutic diet (6). Symptoms of scurvy invariably appeared after 18-24 days and at autopsy extensive haemorrhages and fragility of bones were always observed.

The alkaline phosphatase activity of the following tissues has been studied: serum, bone, intestine, kidney, liver, brain and adrenal. Blood for serum estimation was obtained by cardiac puncture. The extraction of the enzymes from the different tissues and the method for the determination of phosphatase activity has been described before (7).

Results.

The phosphatase activity of the tissues of normal and scorbutic animals are given in Table I. Figures indicate average values for 10-12 animals.

Activity has been expressed in mg of inorganic phosphorus liberated in 1.25 hours per g. of wet tissues and in case of serum per cc.

TABLE I.

	Normal.	Scorbutic.	Fall in activity.
Serum	0.202	0.035	83%
Bone	1.68	0.47	72%
Intestine	8.81	8.36	12%
Kidney	6.05	4.84	20%
Liver	0.260	0.228	18%
Brain	0.560	0.500	10%
Adrenal	1.34	1.18	12%

Discussion.

The phosphatase activity of the different tissues is reduced in scorbutic condition. The degree of decrease is maximum in case of serum and bone, 83% and 72% respectively. The reduction in all other cases is practically of the same order. The figures in each case are the averages of 10-12 determinations, and it was noted that whereas in serum and bone, the degree of reduction of the enzymic activity varied between 69-90% below normal, the phosphatase activity of the other tissues were in some cases not far below normal.

We have previously noted (7) that the response of bone phosphatase to the different dietary factors has a qualitative similarity to that of serum and it is interesting to find that in scorbutic condition the reduction in phosphatase activity of these two tissues is practically same. It has been suggested by some authors (8) that bone is the source of serum phosphatase while according to others (9) phosphatase activity of serum is due to the leakage of the enzyme from tissues like bone, kidney, intestine etc. We found in connection with our studies on the response of phosphatases of different tissues to dietary factors after starvation (7), that while the response of serum and bone phosphatases was qualitatively similar to a single dietary factor the response of serum phosphatase to a mixture of protein, fat and carbohydrate cannot be explained by the simple assumption that serum phosphatase is of osseous origin. We are of opinion that serum phosphatase is of diverse origin.

While ascorbic acid plays such an important part in regulating the phosphatase activity of serum and bone, its influence on the other tissue phosphatases, if present, seems to be very slight.

Summary

Phosphatase activity of different tissues in normal and scorbutic conditions has been studied.

Serum and bone phosphatase activity is markedly lowered in scurvy, while that of the other tissues is affected very slightly. The origin of serum phosphatase has been discussed.

My thanks are due to Dr. B. B. Sircar and Mr. P. B. Sen of the Dept. of Physiology for their interest and encouragement and to the Lady Tata Memorial Trust for a personal grant.

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STUDIES ON CHOLINE-ESTERASE.

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The purification of choline-esterase was first attempted by Stedman and his collaborators from serum (1, 2) and later by Nachmansohn and Ledeser (3, 4) from electric organ of *Torpedo Vulgaris* in artificial sea-water. McMeekin (5) recently obtained a preparation of choline-esterase from serum which is highly purified. Very recently Mendel *et al* (6, 7) report that they have isolated only a very small quantity of a specific enzyme, choline-esterase, from red blood cells which is about twenty times more active than the preparation obtained by Stedman *et al* (2). Their paper, however, does not contain any data or details of the procedure beyond the statement recorded above ; but no attempt has yet been made to isolate it from the venoms of snakes which contain an appreciable quantity of this enzyme. The previous workers (1, 2) proceeded to purify it by fractional precipitation with ammonium sulphate and finally subjected the product to absorption on the surfaces of either aluminium hydroxide or ferric hydroxide sol. in an acidic medium followed by elution with dilute ammonium hydroxide and then with M/3 phosphate buffer of *p*H 8.0. This final product containing the purified choline-esterase has got little action on methyl butyrate and tributyrin.

Measurement of Activity of Choline-esterase :

Different methods have been employed by different workers for the estimation of activity of choline-esterase (8, 9, 10, 11, 12). The choline-esterase activity has been estimated by the author in snake venoms taking advantage of the different bucer solutions to maintain a constant *p*H. The results obtained are in agreement with those obtained by the micromethod using Warburg's apparatus. From this it can be inferred that the buffer solutions in the concentration used in these experiments have no effect on the activity of choline-esterase. The acid liberated is titrated with $N/100$ potassium hydroxide solution in water kept protected from the atmosphere in an atmosphere of nitrogen. *p*H of the reaction mixture is maintained at 7.4 unless otherwise mentioned. The advantage is that the non-enzymatic hydrolysis at this hydrogen ion concentration is negligible while at the optimum *p*H of 8.4 found for this enzyme, this non-enzymic hydrolysis is appreciable. Further the advantages of this procedure involving the use of buffers over others are many. Firstly there is no necessity of adding the indicator at the beginning which inhibits the enzyme action if the indicator is prepared by dissolving in alcohol. Secondly it does not require the tedious procedure of continuous titration matching against solution of definite *p*H. Thirdly wider *p*H ranges are available for the estimation of activity.

Unit of Choline-esterase in Crude Venom :

The previous workers (2) have not clearly defined the unit of choline-esterase obtained from different sources. Their results were generally given on a comparative basis depending upon the weight of the substances whose choline-esterase activity was measured by titrating the acid liberated with sodium hydroxide under definite conditions. To express the results more clearly a term "weight number" has been introduced by them which is defined as the number of cc. of $0.02N$ sodium hydroxide required to neutralise the butyric acid liberated at 30° from butyl choline (used as substrate) in 20 minutes at *p*H 7.4 by the choline-esterase associated with 1 g. of the solid material. The amount of the substrate is 0.25 g. per experiment in a total volume of about 101 cc. The author has also followed the same procedure with some modifications. That amount of cobra venom is taken as equivalent to one unit which hydrolyses in 10 minutes at 37°C 3.97 mg. of acetyl choline chloride at *p*H 7.4 in a total volume of 5 cc. consisting of 2.5 cc of 1% acetyl choline chloride in normal saline, 2 cc. of phosphate buffer of *p*H 7.4 and 0.5 cc. venom solution in physiological saline. The acid liberated in this process is equivalent to 2 cc. of $N/100$ potassium hydroxide. A stream of nitrogen is allowed to pass through the reaction mixture during the titration. It has been found that the rate of hydrolysis is uniform throughout the period of observation, under these conditions. The indicator used is 0.5% phenolphthalein. Units of different venoms found out by this method are represented in the table below:

TABLE I.

Choline-esterase activity of different venoms.

Temp. 37°C Strength of KOH sol. used = N/100.
pH 7.4
Incubation period—10 minutes
Substrate concentration—0.5% acetyl choline chloride.

Type of venom.	Amount of alkali required.	Mg. of acetyl-choline chloride hydrolysed.	Mg. of venom equivalent to one unit of choline-esterase activity.	Unit contained in one gm.
Naja Tripudians (Var. *monocellate*)	2.00 cc.	3.97	1.0	1000
Naja Tripudians (Var. *binocellate*)	2.00 c.c.	3.97	1.0	1000
Bungorus Fasciatus	2.00 cc.	3.97	1.1	909

Determination of Protein : Nitrogen is estimated by the semi-micro Kjeldahl method and the protein ultimately determined by multiplying the nitrogen value by the factor 6.25. The non-protein nitrogen in a solution containing the protein mater is estimated in the supernatant solution obtained after the protein is precipitated with a mixture of 1 cc. of 10% sodium tungstate and 1 cc. of 2/3N sulphuric acid per 10 cc. of the protein solution. After deducting the non-protein nitrogen from the total nitrogen, the protein nitrogen is obtained.

The determination of *p*H is performed with the help of B.D.H. Capillator set unless otherwise mentioned.

Isolation of choline-esterase from the venom of Naja Tripudians (Var. monocellate).

Two grams of the venom are dissolved in 100 cc. distilled water which is adjusted at *p*H 6.0. To this solution is gradually added with constant stirring 22 g. solid sodium sulphate (anhydrous). The mixture is maintained at 35° for 15 minutes and the precipitate is filtered off by means of No. 50 Whatmann filter paper. The precipitate is dissolved in 100 cc. of distilled water and the process of precipitation repeated twice. The final precipitate contains the major portion of the choline-esterase, most of the foreign proteins associated with the crude venom having been eliminated. This precipitate is dissolved in 40 cc. of distilled water adjusted at *p*H 6.0 and treated with 60 cc. of a saturated solution of ammonium sulphate with stirring. The mixture which thus becomes 0.6 times saturated with ammonium sulphate is kept at 0° for 30 minutes. The precipitate is filtered under suction using No. 50 Whatmann filter paper. The precipitate obtained in the preceding stage is again dissolved in 40 cc. of distilled water, the *p*H adjusted at 4.0, and treated with 26 cc. of a saturated ammonium sulphate solution with constant shaking. The mixture is then kept at 0° for 30 minutes and the precipitate is filtered off. The filtrate which contains the greater part of the choline-esterase is again precipitated with the addition of 34 cc. of a saturated solution of ammonium

sulphate which is again placed at 0° for 30 minutes. The precipitate is filtered, dissolved again in 10 cc. of distilled water adjusted at *p*H 6.0, and cooled to 0°. 5 Cc. of the saturated solution of ammonium sulphate are then gradually added to it when a faint permanent turbidity appears. The mixture is then maintained at 0° for four hours after which the precipitate is separated by centrifuging. Weight for weight the activity of this preparation is about 20 times that of the crude venom with which the author started. Although the protein choline-esterase is not obtained in a homogeneous crystalline form nevertheless there is reason to believe that the enzyme obtained according to the above procedure is almost completely free from other active principles and foreign matters present in the cobra venom.

Isolation of Choline-esterase from the Venom of Naja Tripudians (Var binocellate).

By adopting the above procedure in the case of the venom of *Naja Tripudians* (Var. *binocellate*) the choline-esterase preparation attained practically the same degree of purity as was observed in the case of the venom of the *monocellate* variety. Weight for weight the purified product is 19.5 times more active than the original crude venom.

Isolation of Choline-esterase from the Venom of B. Fasciatus.

The peculiarity of the venom belonging to this class of snakes is that although the activity of the choline-esterase in this venom is practically equal to that in the venom of the *monocellate* variety of *Naja Tripudians* yet the purity attained is of a lower order. The final product obtained by adopting the procedure described already is weight for weight only 11 times more active than the crude venom of B. Fasciatus.

Removal of Electrolytes from Choline-esterase.

The precipitate finally obtained by adopting the procedure described already contains besides choline-esterase considerable amount of electrolytes. To remove these the precipitate is dissolved in distilled water and dialysed in a fine cellophane bag of grade No. 100 against distilled water saturated with chloroform. The chloroform-saturated water is used for the purpose of keeping the enzyme solution in sterile condition. The temperature of the dialysing arrangement is maintained at 8° inside a refrigerator. The flow of water is made continuous with the object of getting rid of the salts within a short time. The presence of the salts in the dialysate is tested frequently and when the dialysate becomes completely free from salts the protein solution is withdrawn from the cellophane bag and freed from insoluble matters by centrifuging. The solution is then dried in a vacuum desiccator over fused calcium chloride. The solid choline-esterase thus obtained is dissolved in redistilled water and tested for the presence of salt by means of silver nitrate, barium chloride and Nessler's solution but no indication of chloride, sulphate and ammonium ions is found. This salt-free choline-esterase from the venom of the *monocellate* variety of *Naja Tripudians* has been used in the subsequent experiments unless otherwise mentioned.

TABLE II.

A comparative study of purification of the Choline-esterase from the different Venoms.

Substance.	Per cent of protein.	Per cent of activity.	Purification effected with respect to the crude venom.
Naja Tripudians venom (Var *monocellate*)	91.56	...	...
Naja Tripudians venom (Var *binocellate*)	90.30	...	...
Bungarus Fasciatus venom	98.81	...	...
Choline-esterase (from *Monocellate* variety)	1.11	24	19.7 times.
Choline-esterase (from *Binocellate* variety)	1.20	26	19.5 times.
Choline-esterase (from *B. Fasciatus*)	2.80	27	11.0 times.

TABLE III.

Comparative figures expressed in mg. for the Choline-esterase activity in the purified products obtained from different sources.

Sources.	Unit of choline-esterase (purified).	Units of choline-esterase contained in one gm.
Naja Tripudians venom (Var. *monocellate*)	0.0508 mg.	19,685
Naja Tripudians venom (Var. *binocellate*)	0.0513 mg.	19,493
Bungarus Fasciatus venom	0.1000 mg.	10,000

TABLE IV.

Activity and protein-content of Choline-esterase at various stages in its purification.

Material.	Protein.	Choline-esterase activity.
Crude cobra (var. *monocellate*) *venom*:	91.56%	100%
I. Precipitate from 22% Na_2SO_4	41.13	100
II. Precipitate from 22% Na_2SO_4	21.78	96.5
III. Precipitate from 22% Na_2SO_4	11.91	88.5
IV. Precipitate from 0.6 saturation with $(NH_4)_2SO_4$	7.91	76.5
V. Filtrate after precipitation with 24 cc. of saturated $(NH_4)_2SO_4$	8.67	51.0
VI. Precipitate by adding 36 cc. of saturated $(NH_4)_2SO_4$ to the filtrate	1.94	35.4
VII. Precipitate from $\frac{1}{3}$ saturation with $(NH_4)_2SO_4$	1.11	21.0

Comparative figures of purification of Choline-esterase obtained by different workers starting from different sources :

Stedman *et al* (2) purified the enzyme choline-esterase from normal horse serum whose "weight number" was 100 cc. using butylcholine bromide as substrate. The actual estimation of its activity was made in the following way: To 100 cc. of carbon-dioxide-free water was added 1 cc. of 25% butylcholine bromide and 0.25 cc. of the enzyme solution at *p*H 7.4, the temperature being maintained at 30°. As already mentioned the continuous titration method was followed for measuring the activity. The activity was determined after 20 minutes from the start by titration with 0.02*N* sodium hydroxide. The activity of choline-esterase was also measured by them under the above conditions with acetylcholine chloride as substrate. It was found that under these conditions the hydrolysis proceeds with uniform speed throughout the period of observation. With the object of correlating the rates of hydrolysis using butylcholine bromide (B.C.B.) and acetylcholine chloride (A.C.C.) under the above experimental conditions, some of the comparative data of the previous workers using both the substrates for the same sample of the enzyme are tabulated below:

TABLE V.

Strength of alkali = 0.02N sodium hydroxide.

Temperature 80° Incubation time ... 20 minutes.
Substrate concentration ... 0.217%

Source of serum.	Amount of serum used.	Amount of alkali reqd. using		
		B.C.B. as substrate.	A.C.C. as substrate.	B.C.B./A.C.C.
Monkey	0.25 cc.	8.80 cc.	3.45 cc.	2.55
Horse	0.25 cc.	5.45 cc.	2.00 cc.	2.73
Guinea-pig	0.25 cc.	3.75 cc.	1.35 cc.	2.77
Mean	...	...	...	2.68

The choline-esterase activity in cobra venom has been determined using acetylcholine chloride as substrate in presence of buffers mentioned beforehand. Butylcholine bromide was not available. With the object of comparing the data of the present author with those of the former workers, the choline-esterase activity has been estimated under the conditions of Stedman *et al* using acetylcholine chloride as substrate. To 100 cc. of carbon dioxide-free water is added 1 cc. of 25% acetylcholine chloride and 0.25 cc. of cobra venom containing 0.5 mg. at *p*H 7.4, the temperature being maintained at 30°. With this concentration of the substrate the rate of hydrolysis is found to be uniform during the period of observation. Under these experimental conditions the data obtained for different venoms are tabulated below:

TABLE VI.

Strength of alkali = 0.02*N* sodium hydroxide.

Temperature ...	30°	Incubation time ...	20 minutes.
*p*H ...	7.4	Substrate concentration ...	0.247%

Source of venom.	Amount of venom used.	Amount of alkali required using A.C.C. as substrate.	Calculated value if B.C.B. was used as substrate.
Naja Tripudians (Var *monocellate*)	0.20 mg.	0.50 cc.	1.34 cc.
Naja Tripudians (Var *binocellate*)	0.20 mg.	0.50 cc.	1.34 cc.
B. Fasciatus	0.20 mg.	0.455 cc.	1.22 cc.

The data under the column B.C.B. as substrate are obtained by multiplying the corresponding data under A.C.A. as substrate by 2.68, which follows from Table V.

The former workers (2) obtained in the final stage, a preparation of the enzyme choline-esterase from serum whose "weight number" was 5647. The "weight numbers" for different venoms as well as those for the pure enzyme isolated from these venoms, calculated on the basis of 0.247% concentration of butylcholine bromide as subsrate, are recorded in Table VII for the comparison of the degree of purification attained in the different cases.

TABLE VII

Strength of alkali = 0.02*N* Sodium hydroxide.

Sourse of choline-esterase.	"Weight number" on the starting material.	"Weight number" of the pure enzyme.
Naja Tripudians venom (Var *monocellate*)	6821	124,521
Naja Tripudians venom (Var *binocellate*)	6321	123,260
B. Fasciatus	5746	63,210
Normal horse serum	100	5,647 (Final purification attained by Stedman *et al*).

From Table VII is evident that the enzyme choline-esterase in crude cobra venom is about 64 times more concentrated than that present in the normal horse serum. It will also be noticed that in the final stage of purification the enzyme isolated by the present author from cobra venom is, weight for weight, about 22 times more active than the sample prepared by the previous workers.

Cataphoresis of Choline-esterase isolated from the venom of Naja Tripudians (var. monocellate).

With a view to ascertaining if further purification of the enzyme is possible, cataphoresis in an electric field has been investigated. A stock solution of the

enzyme is mixed with 1 cc. of a buffer solution in a total volume of 10 cc. so that the final concentration is 0.3%. The buffers used in this experiment are phthalate, phosphate and borate according to Sörensen. The experiments are carried out at *p*H 2.2, 3.5, 7.6 and 9.0.

The apparatus consists of a three-chambered glass vessel as shown in Fig. 1. The middle chamber *M* is separated from the two side chambers *B* and *A* by membranes of cellophate No. 400 which allow the enzyme to pass through. The buffered solution of the enzyme (15 cc.) is placed in one of the side chambers while the diluted buffer is placed in each of the remaining chambers. The two side chambers are kept in electrical communication by means of two agar bridges, with copper sulphate solution contained in two beakers. Two copper electrodes are immersed in the two beakers containing the copper sulphate solution. One of the electrodes is connected to the positive pole of the 220 volt lighting circuit through a milliammeter while the other with the negative pole through a key. The whole arrangement is made in such a way that experiments can be conducted inside a refrigerator maintained at 8°. This precaution is taken to prevent the destruction of the enzyme by heat developed during the passage of electric current.

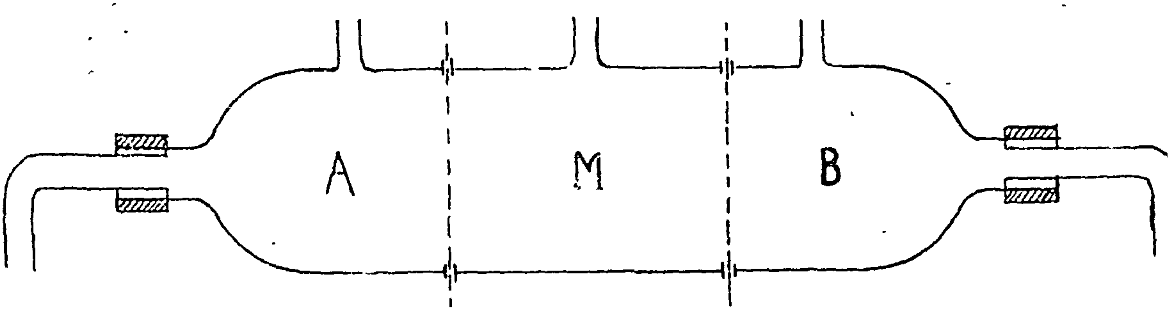

Fig. I

It is to be noted here that at *p*H 2.2 and 3.5, the enzyme solution should be kept in the anodic chamber while at *p*H 7.6 and 9.0, the same should be placed in the cathodic chamber. After the passage of 432 coulombs of electricity through the cell extending over a period of six hours, the contents of the middle and *extreme* chambers such as *M* and *B* are separately examined quantitatively for the enzymic activity and protein contents. The results obtained are tabulated below:

TABLE VIII.

*p*H	Protein content in mg. in chambers.		Units of choline-esterase in chambers.		Units of choline-esterase per mg. of protein in chambers.	
	M	*B*	*M*	*B*	*M*	*B*
2.2	3.01	7.94	59	156	19.9	19.7
3.5	2.30	6.10	45	121	19.6	19.8
7.6	1.98	5.31	38	104	19.6	19.2
9.0	2.57	6.10	51	121	19.8	19.8

Adsorption and Elution of pure Choline-esterase.

As already mentioned, Stedman *et al* (2) attempted to purify choline-esterase by adsorbing it on the surfaces of different suspensions followed by elution ; but the results finally obtained by them were not very encouraging. With the same object in view, 200 units of pure choline-esterase isolated from crude cobra venom are subjected to adsorption by silica, kaolin and ferric hydroxide, 10 cc. of suspension of each of the adsorbents being used in a total volume of 15 cc. Four such reaction mixtures are then kept ready in different tightly stoppered Jena bottles of 25 cc. capacity. These are respectively adjusted to different *p*H such as 2.8, 4.5, 7.6 and 9.4. These bottles containing the solution are shaken mechanically for 30 minutes and the suspension is then separated by contrifuging. The deposit is treated with 10 cc. of 0.10*M* phosphate buffer of *p*H 8.0 and shaken for one hour for elution. The solid matter is centrifuged off and the supernatant liquid, which is freed from foreign ions by dialysis, tested for choline-esterase activity and the protein content by the usual procedures. The dry solid matter in each of the suspensions used is 100 mg. per 10 cc. The results obtained in each case are tabulated below and they show that the further purification of the choline-esterase preparation used is not possible by this procedure .

TABLE IX.

Absorbent used—Silica suspension.

*p*H	Units of choline-esterase.		Total protein in mg. in the elute.	Units of choline-esterase per mg. of protein.
	Amount taken.	Amount eluted.		
2.8	200	67	3.39	19.8
4.5	200	58	2.94	19.7
7.6	200	34	1.72	19.8
9.4	200	31	1.57	19.8

TABLE X.

Absorbent used—Kaolin suspension.

*p*H	Units of choline-esterase.		Total protein in mg. in the elute.	Units of choline-esterase per mg. of protein.
	Amount taken.	Amount eluted.		
2.8	200	56	2.83	19.8
4.5	200	48	2.43	19.8
7.6	200	29	1.45	20.0
9.4	200	26	1.31	19.9

TABLE XI.

Absorbent used—Ferric hydroxide suspension.

*p*H	Units of choline-esterase.		Total protein in mg. in the elute.	Units of choline-esterase per mg. of protein.
	Amount taken.	Amount eluted.		
2.8	200	81	4.10	19.8
4.5	200	73	3.70	19.8
7.6	200	52	2.61	19.9
9.4	200	46	2.30	20.0

Summary and Discussion.

The samples of choline-esterase isolated from the two varieties of *Naja Tripudians* have been purified to such an extent that weight for weight it is about twenty times more active than the starting materials, while that isolated from *Bungarus Fasciatus* venom is weight for weight about eleven-times more active than the crude venom. The degree of purification is practically one-half of what is attained in the case of the venoms of the *Naja Tripudians*. This may be due to the difficult nature of the proteins associated with choline-esterase in the venom of *B. Fasciatus*.

As further purification of choline-esterase isolated from the venom of *Naja Tripudians* (Var *monocellate*) is not possible by means of cataphoretic experiment, it may be supposed that the choline-esterase obtained in the above procedure is a pure sustance. Another additional evidence of its purity may be furnished from the results obtained from the adsorption and elution experiments. These experimental data also show that no further purification is possible. It may be mentioned here that this enzyme isolated from cobra venom is, weight for weight, 22 times more active than that isolated by Stedman *et al* (*loc. cit.*) from serum.

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NICOTINIC ACID, RIBOFLAVIN, ASCORBIC ACID AND GLUTATHIONE CONTENTS OF LIVERS OF SOME CANCER SUSCEPTIBLE (A AND C3H) AND CANCER INSUSCEPTIBLE (C57) STRAINS OF MICE.*

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Experimental evidence is accumulating to suggest that probably the metabolism of liver plays an important rôle both in human cancer and spontaneous tumours in laboratory animals. This is probably associated with the rôle of this organ in the metabolism of sterols, its importance in conversion, detoxication and excretion of many harmful substances, its relationship to bile acids and of the latter to some known carcinogenic substances. This belief has been further strengthened by the investigations of Des Ligneris (1), Kleinenberg, Neufach and Shabad (2), Neufach and Shabad (3) on human subjects and of Selle and others (4) on laboratory animals susceptible to cancer.

*The work was undertaken under the direction of Dr. V. R. Khanolkar, the Director of Laboratories.

It was, therefore, logical to believe that if cancer susceptibility was related to liver metabolism homozigous starins of mice having varying degree of susceptibility to spontaneous tumours would show some variations in their liver metabolism. Thus Figg and others (5) found the xanthine-oxidase activity of livers of certain strains of mice susceptible to carcinoma of the mammary gland (C3H) was far less than that of the other resistant strain (JK). Khanolkar and Chitre (6) and Chitre and Khanolkar (7), from their studies on blood, liver, and excretion of esterase in cancer-susceptible (C3H and A) and cancer-resistant strain (C57), suggested that probably the liver metabolism differed in these two types of strains. An investigation in this direction was thought worth pursuing and to start with the estimation of nicotinic acid, riboflavin, vitamin C and glutathione content of livers of such animals was undertaken.

EXPERIMENTAL

Material.—During the investigation three inbred strains of mice having varying susceptibility of spontaneous mammary cancer were used. (*i*) Strain C57 is highly resistant to cancer and the incidence of spontaneous mammary cancer is reported to be as low as 1% (Andervont, 8). (*ii*) Strain C3H is highly susceptible to spontaneous breast tumours. In this strain both breeding and non-breeding females develop the spontaneous tumour to the extent of 95 to 100%. (*iii*) Strain A is such that breeding females develop the tumours to the extent of 80 to 85% while the incidence in virgins is only 4.5% (Andervont *et al*, 9).

The animals were getting a balanced food in the form of dried pellets used at this hospital for the last three years. In addition to pellets the mice received fresh vegetables and fresh whole milk about 2cc. per mouse every day. The water was given *ad lib*. The young animals were allowed to wean after four weeks. The males and females were kept in separate cages. When the mice were between 6 and 8 months old they were sacrificed under chloroform anesthesia. The livers were immediately removed and weighed. The above estimations were made on aliquot positions by the methods given below.

Methods.—(*a*) *Determination of Nicotinic acid*: The method of estimation is as below: (*i*) The hydrolysis and extraction of the acid by 2*N*-HCl. (*ii*) Adjustment of *p*H by sodium hydroxide to 6.5-7. (*iii*) Subsequent separation of coagulating material and colorimetric estimation by using cyanogen bromide and aniline hydrochloride as the reagents.

A sample of liver tissue weighing about 0.5-0.6 g. was accurately weighed in a weighing bottle. The sample was taken up in a glass mortar and triturated with fine glass powder. The mixture was transferred to a pyrex text tube using small amounts of distilled water for washing. The total volume of such suspension was made to 8cc. and 2cc. of hydrochloric acid (conc.) were added. It was then heated to slow boiling for about 30 minutes under reflux to avoid loss by evaporation. The contents of the tube were removed to a 15 cc. centrifuge tube. The test tube was washed with small portions of distilled water and the washings were added to

the original solution. It was then centrifuged for 10 minutes at 3000 R.P.M. and the supernatant liquid was carefully separated. The *p*H of this solution was adjusted to 6.5-7 by means of sodium hydroxide and slightly warmed. Small amount of precipitate which separated out was filtered off through a wet filter paper. The clear filtrate corresponding to 0.2 gm. of the tissue was used for colorimetric estimation.

The above aliquot filtrate was kept in water-bath maintained at 70° for a few minutes. 2 Cc. of cyanogen bromide were added to it and the contents were well mixed. The tube was allowed to stand in water-bath for further few minutes and then rapidly cooled under tap water. 2 Cc. of aniline hydrochloride (10%) were then added and the development of colour was allowed to take place in dark for further 5 minutes. Intensity of the colour was then measured on Pfaltz-Bauer fluoro-photometer using filter combination having peak at 4500 Å. The quantity of nicotinic acid was calculated from the reference graph drawn under similar conditions with pure nicotinic acid. (*b*) *Fluorometric estimation of riboflavin :* Sample of tissue (0.8-1 g.) was weighed out accurately and ground in a mortar with small amount of glass powder. The mass was transferred to a test tube with the addition of 20 cc. of 0.3% pepsin solution in *N*/20 HCl. Few drops of toluene were added as a preservative and the mixture was incubated at 37° for 24 hours. It was then slightly warmed and centrifuged. The supernatant liquid was poured out carefully. 0.5 Cc. of 3% potassium permanganate was added and shaken vigorously. The excess of permanganate was decolorised by adding hydrogen peroxide drop by drop. To the mixture was then added 1 cc. of 40% trichloroacetic acid and turbidity that resulted was removed by filtration. The volume of the final filtrate was noted. Estimation of riboflavin was done by measuring the fluorosence of the solution on Pfaltz-Bauer fluorophotometer using filter combination of blue, orange and yellow filters having peak at 4400 Å. The blank reading was obtained by destroying the riboflavin by exposure to sunlight for 10 hours. From these readings the riboflavin content was found by referring to a reference graph plotted by using pure riboflavin.

(*c*) *Estimation of ascorbic acid :* The method followed during the course of the investigation was based on that described by Mindlin and Butler (10). 2.5% Sulphosalycilic acid was however used instead of metaphosphoric acid, as the latter was not available in large amounts needed for the experiment. About 0.5 g. of tissue was titurated and the final volume was made up to 15 cc. of 2.5% acid and filtered. Aliquot parts of the filtrate were used for the estimation of ascorbic acid and glutathione.

The indicator, 2:6-dichlorophenol-indophenol, was prepared by dissolving 1 tablet of B.D.H. tabloid preparation in 100 cc. of distilled water. At the time of measuring the reduction of dye on Pfallz-Bauer fluorophotometer 4 cc. of the dye and 2 cc. of the unknown protein-free filtrate were used. Dye blank was taken by using 2 cc. of 2.5% sulphosalycilic acid instead of the unknown solution. Reagent blank was taken by decolorizing the above solution by ascorbic acid to make the necessary correction for impurities. Ascorbic acid was found by referring to a reference graph drawn by plotting the (*log* Gs.-*log* Gb.) against the percentage of

but the findings do not suggest any definite relationship between the susceptibility of the strains to spontaneous breast cancer and the various contents of the liver. It is however too early to draw any conclusion as to the interrelation between the liver metabolism and cancer susceptibility in mice on the basis of such investigations in which only few factors have been studied. It may be probable that the factors studied here may be of secondary importance and may not give any direct indication as to the relation between the susceptibility of the strain to the spontaneous tumours and their liver metabolism. Further investigations are, therefore, essential to throw more light on this aspect of the problem.

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